# THE MOLECULAR BASIS OF DEMETHYLATION INHIBITOR FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA* AND THE ROLE OF SEED INOCULUM IN CERCOSPORA LEAF SPOT DISEASE OF SUGAR BEET

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Cercospora leaf spot (CLS) is the most destructive foliar disease of sugar beet worldwide. CLS is caused by the filamentous fungus *Cercospora beticola*. Disease management currently relies upon timely application of fungicides, but reliance on certain chemical classes has led to the development of resistance in multiple *C. beticola* populations. One such class is the demethylation inhibitor (DMI) fungicides of which the genetic basis of resistance has been unclear. Therefore, the first objective of this PhD research was to perform a genome-wide association study on 190 *C. beticola* isolates to identify mutations associated with tetraconazole (a common DMI) fungicide resistance. Whole genome resequencing identified multiple novel loci associated with sensitivity to tetraconazole including a pleiotropic drug resistance ATP-binding cassette transporter, a regulator of G-protein signaling domain (RGD) protein, a DYRK protein kinase and mutations within and upstream of the gene encoding the DMI target Cytochrome P450 51 (CYP51). This demonstrated the genetic complexity in resistance to DMIs and suggested the involvement of cellular signaling and multidrug resistance as well as target site mutations. The second objective of this research was to investigate the potential of seedborne *C. beticola* to initiate CLS disease in sugar beet. We showed that viable *C. beticola* was present in commercial sugar beet seed lots and could function as primary inoculum to cause CLS symptoms in seedlings. All strains identified were resistance to QoI fungicide chemistries and most were also resistant to DMI fungicides. Detection of *C. beticola* DNA in xylem sap suggested that the fungus may be systemically colonizing the plant via the vascular system. Long-read nanopore sequencing detected other potential pathogenic fungi in seed DNA (e.g. *Fusarium* and *Alternaria* spp.) that may also act as primary inoculum sources for important sugar beet diseases. This PhD research has improved our understanding of the development of DMI fungicide resistance in *C.*

*beticola*, as well as highlighted the importance of seed inoculum in the manifestation of CLS in sugar beet, both of which could improve CLS disease management in the future.

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# CHAPTER 1. *CERCOSPORA BETICOLA:* THE INTOXICATING LIFESTYLE OF THE LEAF SPOT PATHOGEN OF SUGAR BEET[1]

## 1.1. Summary

Cercospora leaf spot, caused by the fungal pathogen *Cercospora beticola*, is the most destructive foliar disease of sugar beet worldwide. This review discusses *C. beticola* genetics, genomics and biology and summarizes our current understanding of the molecular interactions that occur between *C. beticola* and its sugar beet host. We highlight the known virulence arsenal of *C. beticola* as well as its ability to overcome currently used disease management strategies. Finally, we discuss future prospects for the study and management of *C. beticola* infections in the context of newly employed molecular tools to uncover additional information regarding the biology of this pathogen.

Taxonomy: *Cercospora beticola* Sacc.; Kingdom Fungi, Phylum Ascomycota, Class Dothideomycetes, Order Capnodiales, Family Mycosphaerellaceae, Genus *Cercospora*.

Host range: Well-known pathogen of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) and most species of the *Beta* genus. Reported as pathogenic on other members of the Chenopodiaceae (e.g. lamb's quarters, spinach) as well as members of the Acanthaceae (e.g. bear's breeches), Apiaceae (e.g. *Apium*), Asteraceae (e.g. chrysanthemum, lettuce, safflower), Brassicaceae (e.g. wild mustard), Malvaceae (e.g. *Malva*), Plumbaginaceae (e.g. *Limonium*) and Polygonaceae (e.g. broad-leaved dock) families.

[1]The material in this chapter was co-authored by Rebecca E. Spanner, Lorena I. Rangel, Malaika K. Ebert, Sarah J. Pethybridge, Eva H. Stuckenbrock, Ronnie de Jonge, Gary A. Secor and Melvin D. Bolton. Rebecca E. Spanner, Lorena I. Rangel and Malaika K. Ebert had primary responsibility for writing the review, making figures, and editing sections from other contributors. Sarah J. Pethybridge wrote the population genetics section, Eva H. Stuckenbrock wrote the population genomics section and Ronnie de Jonge contributed the genome analysis section. Gary A. Secor and Melvin D. Bolton served as proofreaders.

Disease symptoms: Leaves infected with *C. beticola* exhibit circular lesions that are colored tan to grey in the center and are often delimited by tan-brown to reddish-purple rings. As disease progresses, spots can coalesce to form larger necrotic areas causing severely infected leaves to wither and die. At the center of these spots are black spore-bearing structures (pseudostromata). Older leaves often show symptoms first and younger leaves become infected as the disease progresses.

Management: Application of a mixture of fungicides with different modes of action is currently performed although elevated resistance has been documented in most employed fungicide classes. Breeding for high-yielding cultivars with improved host resistance is an ongoing effort and prudent cultural practices, such as crop rotation, weed host management and cultivation to reduce infested residue levels, are widely used to manage disease.

### 1.2. Domestication history of *Beta vulgaris* and the impact of *C. beticola* on modern farming

The world currently relies on sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) for approximately one-fifth of its total sugar (ISO, 2018). While the other major source of sucrose, sugar cane, is grown in more tropical climates, sugar beet is largely grown in temperate regions with the northern tier states in the U.S., France, Germany and Russia being the top producers (FAOSTAT, 2019). All cultivated beets (fodder, chard, table beet, sugar beet) are regarded to have derived from wild Mediterranean sea beet (*B. vulgaris* ssp. *maritima*) (OECD, 2001). In the late 18$^{th}$ century, the German chemist Andreas Marggraf showed that sucrose in both white and red beetroot was chemically identical to the prohibitively expensive tropical cane sugar (Marggraf, 1748). Marggraf's student Franz Karl Achard noticed that conical white fodder beets had high sugar content and began to grow them for sugar production (Fischer, 1989). Achard is credited with establishing the sugar beet industry in Europe by building the first processing factory in Silesia (now Poland) in 1801. Sugar beet was subsequently commercialized in the U.S.

in 1879 (Magnuson, 1918). Continuous breeding over the last 200 years has led to an increase in sugar content from 8% to more than 18% in cultivars grown today (Dohm et al., 2014). Additionally, the discovery of male sterile cytoplasm permitted the development of hybrid varieties for yield increase (Biancardi et al., 2010). Although breeding and improved cultivation practices yielded significant increases in sugar beet production and sugar yield over the last decades, many abiotic and biotic stresses remain that continue to affect sugar beet growth and ultimately attenuate sugar production worldwide.

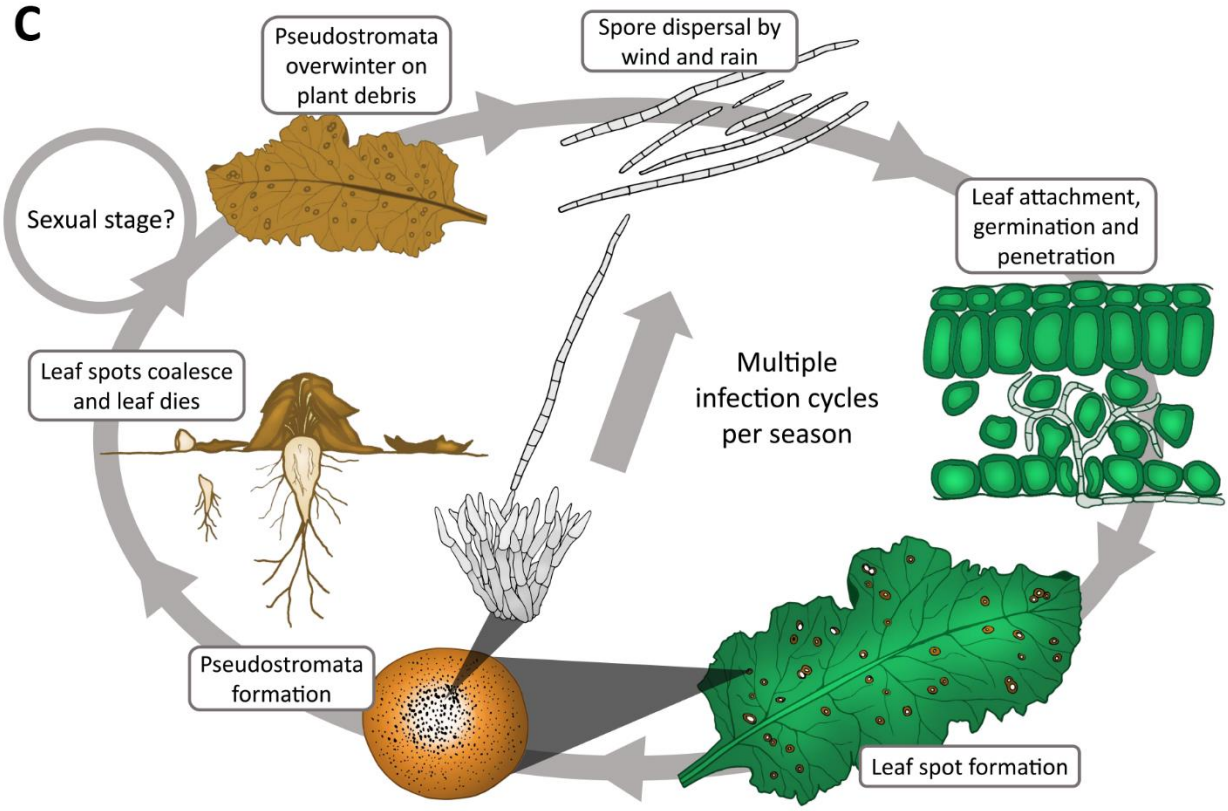
The most common and destructive foliar disease of sugar beet globally is Cercospora leaf spot (CLS) (Holtschulte, 2000). The disease was first described on *B. cicla* in Italy by Saccardo (1876) but now has been identified across the globe wherever sugar beet is grown. CLS is most pernicious in warm, humid growing regions (Lartey et al., 2010), which make up almost one-third of the total sugar beet production area in the U.S (USDA-NASS, 2010). The main adversity is the loss of recoverable sucrose, reaching almost 50% under uncontrolled moderate to high disease pressure (Lamey et al., 1987; Shane and Teng, 1992). Additional economic losses occur as a result of increased impurities that complicate sucrose recovery processes, leading to higher processing costs and reduced extractable sucrose (Shane and Teng, 1992). Diseased plants are also more susceptible to storage rot in winter storage piles (Smith and Ruppel, 1973). Large economic losses were seen in sugar beet crops in Southern Germany in the late 1980s and early 1990s due to severe CLS epidemics (Wolf and Verreet, 2005). In the U.S., Minnesota and North Dakota suffered losses from CLS in 1998 estimated at $113 million from yield reduction and fungicide application costs (Cattanach, 1999). In these areas, with conducive environmental conditions, it is crucial to appropriately apply fungicides otherwise substantial loss of foliar photosynthetic area can occur due to CLS (Fig. 1.1A & B). The upper Midwest has seen

epidemics in three out of the last four years in large part due to fungicide resistant *C. beticola* populations that have resulted in several hundred million dollars in lost revenue (Mike Metzger, Minn-Dak Farmers Cooperative, *personal communication*). A deeper understanding of the *C. beticola*-sugar beet interaction may allow for the development of innovative strategies to prevent the disease and ultimately cut much of these losses. Here, we discuss the biology of this pathogen and explore the recent advances in the molecular and genetic understanding of CLS.

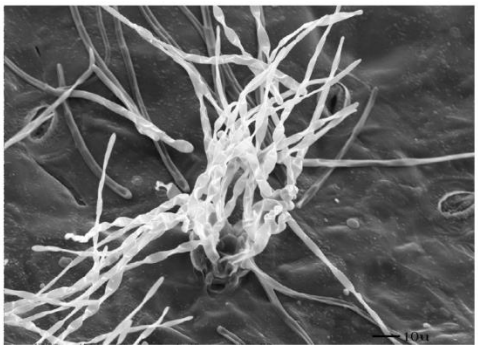
A

B

C


Pseudostromata overwinter on plant debris
Spore dispersal by wind and rain
Sexual stage?
Leaf attachment, germination and penetration
Leaf spots coalesce and leaf dies
Multiple infection cycles per season
Pseudostromata formation
Leaf spot formation

D



E

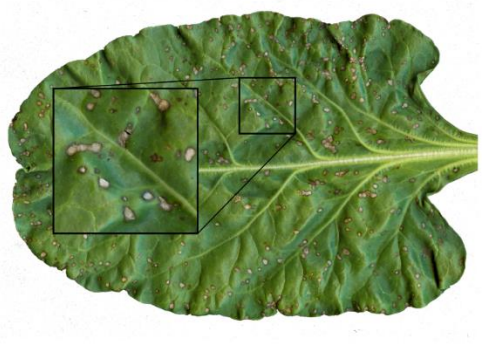


Figure 1.1. Cercospora leaf spot (CLS) of sugar beet. A) Drone image highlighting the importance of fungicides for disease management. While the disease was well-managed on the right side of the field, an abrupt halt in fungicide application ultimately resulted in increased CLS disease as evidenced by the brown color noted on the left side of the field. B) Extensive CLS disease in a sugar beet field. C) Disease cycle of *Cercospora beticola* on sugar beet. Infection is initiated by airborne or splash-dispersed conidia that penetrate the sugar beet leaf through stomata and give rise to intercellular hyphal growth. Leaf spots form on the leaves after the switch to necrotrophy, which typically occurs seven days post-infection. Pseudostromata develop in these lesions and asexually produce spores, leading to multiple infection cycles during the growing season. The pseudostromata are also the overwintering structures on plant debris at the end of the season. It is possible that *C. beticola* can sexually reproduce and produce ascospores, similarly to *Zymoseptoria tritici*, but this stage has not been observed. D) Scanning electron micrograph exhibiting C. beticola conidiophores emerging from sugar beet. E) CLS symptomology on sugar beet.

### 1.3. Infection biology

The causal agent of CLS, *C. beticola*, has the ability to complete several asexual cycles within a single season under conducive conditions (McKay and Pool, 1918; Nagel, 1945; Vereijssen et al., 2007). Between sugar beet growing seasons, the fungus survives primarily as desiccation-resistant hyphal structures on infected plant residues within leaf substomatal cavities. These specialized overwintering structures are known as pseudostromata, or false stromata (conidia-producing hyphae), since they are comprised of both fungal tissue and remnants of host tissue (Eriksson, 1981). Pseudostromata can persist on plant debris for two years and have long been regarded as the primary inoculum sources for infection (Khan et al., 2008; Pool and McKay, 1916). However, many population studies have questioned the role of clonally reproducing primary inoculum (Groenewald et al., 2006; Groenewald et al., 2008; Knight et al., 2018; Knight et al., 2019). Other potential sources of initial inocula include dispersal of *C. beticola*- infested plant material via tools or machinery (Knight et al., 2018; Knight et al., 2019), infested seed, windborne conidia, or stromata from other host plants (Franc, 2010; Khan et al., 2008; Knight et al., 2020; Skaracis et al., 2010; Tedford et al., 2018).

*C. beticola* is an ascomycete fungus only known to exist in the anamorphic (asexual) state (Crous et al., 2001; Groenewald et al., 2013). To reproduce, melanized conidiophores form from pseudostromata to produce conidia in the spring but this may be preceded by saprophytic, vegetative growth of fungal mycelia (Pool and McKay, 1916) (Fig. 1.1C). Transmission electron microscopy has shown that bundles of 10-20 conidiophores (18-25µm in diameter) often form on both upper and lower leaf surfaces and are produced from subepidermal or substomatal pseudostromata (Pons et al., 1985). Pseudostromata are 3-6 cells deep and up to 8-10 cells wide. Conidiophores consist of 1-2 cells and are 10-25µm long × 3-5µm wide at the base (Fig. 1.1D). Conidia are needle-shaped (2-3 × 36-107µm) and colorless with several cross-walls (Weiland and Koch, 2004). The minimum requirements for conidial development are temperatures of at least 15°C and a relative humidity of 60% or higher (Pool and McKay, 1916; Solel and Minz, 1971a). Through wind, rain, irrigation, water splash or insect transfer, spores are disseminated and make contact with the abaxial surface of sugar beet leaves or petioles to initiate infection (Khan et al., 2007; Lawrence and Meredith, 1970). Some studies have suggested that roots may also act as primary infection sites (Vereijssen et al., 2005), although the specific requirements for root infection remain elusive (Khan et al., 2008). Germination of conidia is optimal at high relative humidity (near 100%) and at temperatures of approximately 25°C (Khan et al., 2009; Ruppel, 1986). After germination, appressoria are produced allowing hyphae to penetrate leaf tissue through stomata and spread intercellularly with no visible leaf symptoms (Rathaiah, 1977; Steinkamp et al., 1979). As the fungus switches to its necrotrophic phase, the production of phytotoxins and degradative enzyme activity leads to the necrotizing of infected cells (Steinkamp et al., 1979). Symptoms appear as circular spots 3-5 mm in size and tan to gray in color, encircled by a tan-brown to red-purple border (Windels et al., 1998) (Fig. 1.1E). Symptoms can

develop on older leaves as quickly as five days after infection when there are favorable conditions of high humidity (>90%) and warm temperatures (day 27-32°C, night >17°C) (Pool and McKay, 1916; Solel and Minz, 1971b). In the field, these characteristic CLS lesions are typically observed after sugar beet canopy closure (Khan et al., 2008). Pseudostromata formed within the lesions become the site for production of new conidia as early as seven days post-infection under favorable conditions (Jacobsen and Franc, 2009). Conidia are again disseminated by wind, rain splash or insect transfer to initiate another infection cycle. Early studies show evidence for rain splash being the major factor in spore dispersal (Carlson, 1967) while studies by Khan et al. (2008) suggested that wind was the major dispersal factor for *C. beticola* inoculum, since higher disease severity was observed on exposed plants when compared to plants in plastic cages or with ground cover. The distance that conidia can travel whilst retaining viability has not been reported, but genetic studies have provided evidence that long-range dispersal has occurred (Groenewald et al., 2008; Knight et al., 2018; Vaghefi et al., 2017a).

### 1.4. Population genetics

The population biology of *C. beticola* has been extensively explored to quantify genetic structure, gene flow, and genotypic and genetic diversity in space and time. Tools used to study *C. beticola*'s evolutionary ecology have included random amplified polymorphic DNA, amplified fragment length polymorphisms, microsatellites, and single nucleotide polymorphisms (Groenewald et al., 2007; Turgay et al., 2010; Vaghefi et al., 2017b). A consistent theme in these studies has been to provide indirect evidence for sexual recombination, migration, and mutation. These findings have supported the development of hypotheses surrounding pathogen biology and movement with important implications for disease epidemiology and management. For example, the lack of a known teleomorph for *C. beticola* means a substantial gap exists in the knowledge

surrounding primary inoculum sources and between-field inoculum movement (Pethybridge et al., 2018).

*C. beticola* populations are generally characterized by high allelic, genetic, and genotypic diversity, typically coinciding with remarkably high phenotypic diversity (Moretti et al., 2004; Ruppel, 1972) that is often exhibited in *in vitro* colony morphology (Fig. 1.2). Although *C. beticola* is heterothallic (self-sterile) where any one isolate has either mating type (*MAT*) gene (*MAT1-1-1* or *MAT1-2-1*) at the *MAT1* locus, additionally each isolate has *MAT1-1-1* and *MAT1-2-1* exon fragments located at ostensibly random loci across the genome (Bolton et al., 2014). Interestingly, these *MAT* fragments could largely reconstitute a hypothetical fused organization of *MAT* genes, suggesting a homothallic (self-fertile) ancestral state for *C. beticola* and other related species (Bolton et al., 2014). Ratios of isolates with different mating types (MAT1-1 and MAT1-2) within populations have been location-specific. Isolates of one mating type have dominated populations from sugar beet in Iran (Bakhshi et al., 2011) and one production region within the U.S. (Obuya et al., 2011). In contrast, mating type ratios in equilibrium have been characterized in populations from sugar beet fields in Minnesota and North Dakota (Bolton et al., 2012c), and table beet in New York (Vaghefi et al., 2017c). Equal mating type ratios together with high genotypic diversity have supported the potential for sexual recombination within populations (Bolton et al., 2012c; Groenewald et al., 2006; Groenewald et al., 2008). Additionally, microsatellite alleles in linkage disequilibrium when combined with equal mating type ratios and high genotypic diversity in certain *C. beticola* populations from table beet provides evidence to suggest that a mixed reproductive mode is more likely (Knight et al., 2018; Vaghefi et al., 2016; Vaghefi et al., 2017c).

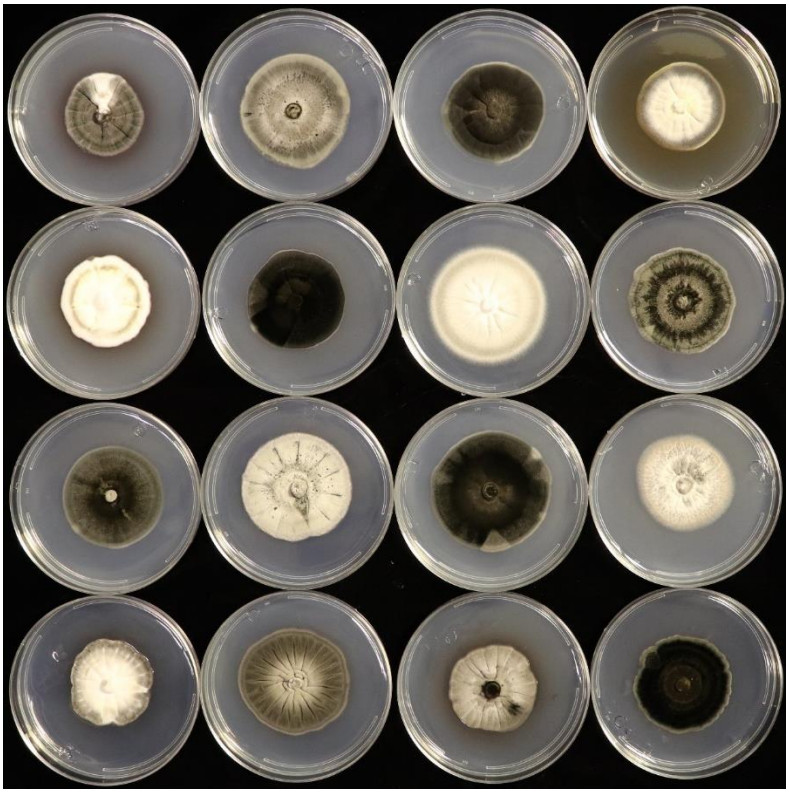


Figure 1.2. Phenotypic diversity among *Cercospora beticola* isolates sampled from fields in the Red River Valley. All isolates were grown on potato dextrose agar for 14 days at room temperature under constant light.

Several studies have also quantified genetic homogeneity and genetic differentiation between *C. beticola* populations on variable spatial scales ranging from different hosts in the same field (Vaghefi et al., 2017c), fields within and between regions (Vaghefi et al., 2016; Vaghefi et al., 2017b), and between continents (Groenewald et al., 2008; Knight et al., 2019; Vaghefi et al., 2017a). High levels of gene flow and low differentiation between *C. beticola* populations on different continents have supported evidence for genetic similarity and panmictic populations and the potential role of infested plant material dissemination in pathogen movement and initiation of epidemics (Groenewald et al., 2008; Knight et al., 2019; Vaghefi et al., 2017b). Knight et al. (2019) reported genetic diversity, differentiation and relationships among 948 *C. beticola* isolates from 28 populations in eight regions and identified two major clusters. One

cluster was specific to New York, U.S. with evidence of recent expansion and divergence; and another common across North and South America, Eurasia, Hawaii, and selected states within the U.S. (North Dakota and Michigan) with shared origin (Knight et al., 2019). Human-mediated dispersal of *C. beticola*- infested plant material may be responsible for primary inoculum within individual seasons, while findings from studies that evaluated the temporal stability of populations within fields have challenged the role of overwintering inoculum in epidemic initiation (Knight et al., 2018; Knight et al., 2019).

### 1.5. Population genomics

Genome sequencing has provided valuable insight into the evolution and biology of diverse fungal plant pathogens (Soanes et al., 2007). Genome sequencing of *C. beticola* was initially aimed to identify new polymorphic markers for genotyping of field isolates (Vaghefi et al., 2017b), and unravel biosynthetic gene cluster evolution (de Jonge et al., 2018).

The genome size of *C. beticola* is comparable to other closely related Dothideomycetes, however the repeat content is considerably lower and includes less than two percent of the entire genome (de Jonge et al., 2018). In comparison, genomes of the two related species *Mycosphaerella fijiensis* and *Cladosporium fulvum* belonging to the same order Capnodiales have a significantly higher repeat content at 37% and 44%, respectively (Ohm et al., 2012). This indicates the presence of efficient genome defense mechanisms in *C. beticola* such as repeat induced point mutations and DNA methylation that may prevent the propagation of repetitive elements (Cambareri et al., 1989; Gladyshev, 2017).

Population genomic sequencing of some other fungal plant pathogens, including *Leptosphaeria maculans* and *Zymoseptoria tritici*, have demonstrated that repetitive elements can associate with presence/absence variation of genes (Plissonneau et al., 2016; Rouxel et al., 2011). Despite the low repeat content in *C. beticola* (de Jonge et al., 2018), recent analyses of

population genomic data have revealed a substantial number of genes showing presence/absence variation (Spanner and Bolton, *unpublished*). It is possible that a dynamic repertoire of effector genes is driven by a coevolutionary arms race between plant and pathogen and facilitates the infection of *C. beticola* on different hosts.

We analyzed North American field populations of *C. beticola* to assess the amount and distribution of genetic variation and infer about the recent demography of the pathogen. When analyzing re-sequencing data from more than 100 field isolates, we identified approximately 500,000 SNPs from which we computed a mean nucleotide diversity of $\pi = 0.0035$ (Potgieter, Bolton and Stukenbrock, *unpublished*.). We found that $\pi$ varies considerably along the *C. beticola* genome including regions with a ten-fold increase in genetic variation. Further analyses should scrutinize patterns of genetic variation along the genome to understand the functional relevance of variation "hotspots" and the underlying drivers of genetic variability. Research in genome evolution of *C. beticola* could have great importance in the development of new management strategies, including in the identification of new fungicide targets.

Population genomic data can also clarify the demographics within a species (Dutheil and Hobolth, 2012; Excoffier et al., 2013). Different demographic scenarios such as population bottleneck and a population expansion impact the distribution of genetic variation in a population. A population bottleneck will significantly reduce genetic variation in the population while a population expansion will lead to an excess of newly derived mutations. Different measures, derived from the distribution of allele frequencies along the genome, reflect recent demographic events that have affected genetic variation in a population (Alcala et al., 2013). We used the population genomic data of *C. beticola* to compute the Tajima's D statistic (Tajima, 1989) along the genome to measure genetic variation at the DNA level via a standardized

pairwise comparison of the number of nucleotide differences to the number of segregation sites. While the value varies significantly along chromosomes, we find an overall positive Tajima's D value of 1.1. A Tajima's D value > 0 reflects an excess of alleles of intermediate frequencies, which is expected under a scenario of population contraction. While additional analyses are required to infer the recent demography of *C. beticola* in North America, this positive Tajima's D value could indicate a recent loss of genetic variation in the pathogen population due to demographic changes. Recent demographic changes in populations of *C. beticola* may result from selection on the fungus imposed by fungicide treatment and resistant beet cultivars. It may also reflect the recent emergence of the pathogen on sugar beet as a new host. Comparative population genomic analyses can provide an opportunity to date different demographic events that have shaped genetic variation in the pathogen.

Population genomic sequencing of *C. beticola* from other continents and from wild hosts will furthermore provide important information into the center of origin and the global population genetic structure of the pathogen, as well as patterns of gene flow and signatures of selection in agricultural and wild ecosystems.

### 1.6. Development and characterization of the *C. beticola* reference genome

#### 1.6.1. *C. beticola* genome statistics

We previously reported the *C. beticola* reference genome size to be 37 Mbp (de Jonge et al., 2018). The final, high-quality annotation contained 12,281 genes and 12,495 transcripts, encompassing 28,389 unique exons. On average, protein-coding genes contained 2.3 exons and 1.3 introns that were approximately 780 and 73 bp long, respectively. A significant proportion of genes (4,384 genes or 35.7%) did not contain an intron and are referred to as single-exon genes. We assigned both 5' untranslated regions (UTRs) and 3' UTRs to 7,147 genes, 5' or 3' UTR to 1,561 genes and 3,573 genes did not have an assigned UTR. The average length of the 5' UTR

was 188 bp and that of the 3' UTR 300 bp. Most intronic sequences were found in the coding regions (15,195 or 96%). We identified 446 introns (2.8% of all introns) in 5' UTRs and 181 (1.2% of all introns) in 3' UTRs, representing a significant under-representation of 3' UTR introns exemplified by the 25-fold lower intron density ($7.6 \times 10^{-5}$ introns/mRNA transcripts) as compared to 5' UTR introns ($3.0 \times 10^{-4}$ introns/mRNA transcripts). In *Arabidopsis* and some mammals, a pronounced under-representation of 3' UTR introns may be explained by a requirement for the nonsense-mediated decay (NMD) pathway (Chung et al., 2006; Lejeune and Maquat, 2005). NMD is a cellular process that is involved in the detection and decay of mRNA transcripts that contain premature stop codons. Our results suggest that filamentous fungi may utilize a similar NMD pathway. In this regard it is interesting to note that an examination of the *C. beticola* proteome resulted in the identification of three important protein components of the exon junction complex which is essential for NMD (Lejeune and Maquat, 2005). Examination of UTR characteristics in other related fungi based on available gene structures supports this view, albeit limited by the number as well as the accuracy of these gene structures.

A detailed overview of all gene model statistics, that were previously reported in de Jonge et al. (2018), is shown in Table 1.1 as well as a comparison of these statistics with five related plant pathogenic Dothideomycete fungi with well-characterized genome sequences. These are *Dothistroma septosporum* (teleomorph *Mycosphaerella pini*), *Z. tritici* (previously known as *Mycosphaerella graminicola*), *L. maculans*, *Parastagonospora nodorum* (previously known as *Stagonospora nodorum*) and *Pyrenophora tritici-repentis* as well as a more distantly related Eurotiomycete *Aspergillus nidulans*.

Table 1.1. Cercospora beticola genome statistics and comparison to other related fungi.

| **Species*** | **Cbe** | **Cbr** | **Ccn** | **Dse** | **Ztr** | **Lma** | **Ptr** | **Pno** | **Ani** |
|---|---|---|---|---|---|---|---|---|---|
| **Assembly statistics** | | | | | | | | | |
| Total assembly length (Mbp) | 37.1 | 37.4 | 34.0 | 30.2 | 39.7 | 44.9 | 37.8 | 37.2 | 30.5 |
| Total length of gaps (Mbp) | 3.2 | 0.3 | 0.0 | 0.1 | 0.0 | 1.1 | 0.6 | 0.2 | 0.7 |
| No. of scaffolds/contigs | 248 | 28,905 | 6,126 | 20 | 21 | 41 | 47 | 108 | 8 |
| NG50 scaffolds (No.) | 4 | 111 | 422 | 5 | 6 | 10 | 6 | 13 | 4 |
| LG50 scaffolds (Mbp) | 4.17 | 0.096 | 0.023 | 2.60 | 2.67 | 1.77 | 1.99 | 1.05 | 3.76 |
| NG95 scaffolds (No.) | 10 | 11,497 | 2,320 | 12 | 18 | 25 | 20 | 43 | 8 |
| GC content (%) | | | | | | | | | |
| Overall (excl. gaps) | 52.2 | 51.5 | 52.6 | 53.1 | 52.1 | 45.2 | 51.0 | 50.4 | 50.4 |
| Coding (CDS) | 53.8 | 53.9 | 54.3 | 54.6 | 55.6 | 54.1 | 53.6 | 54.6 | 53.4 |
| Repeat content (Mbp) | 0.51 | ND*** | ND*** | 1.08 | 6.98 | 15.93 | 0.80 | 2.88 | 1.07 |
| **Protein-coding genes** | | | | | | | | | |
| Protein-coding genes (No.) | 12,281 | 11,972 | 11,556 | 12,580 | 10,951 | 12,469 | 12,169 | 12,380 | 10,680 |
| Mean gene length (bp) | 1,885 | 1,584 | 1,556 | 1,896 | 1,602 | 1,446 | 1,616 | 1,468 | 1,736 |
| Percentage coding | 68.4 | 51.1 | 52.9 | 79.3 | 44.2 | 41.2 | 52.9 | 49.1 | 62.2 |
| Mean gene density (No. genes/100 Kbp) | 36.3 | 32.3 | 34.0 | 41.8 | 27.6 | 28.5 | 32.7 | 33.4 | 35.8 |
| Mean CDS length (bp) | 1,469 | 1,473 | 1,450 | 1,223 | 1,310 | 1,258 | 1,349 | 1,271 | 1,456 |
| Exons** | | | | | | | | | |
| No. of exons | 28,100 | 28,046 | 26,848 | 28,937 | 28,611 | 35,201 | 32,716 | 32,994 | 34,743 |
| Exons/gene | 2.3 | 2.3 | 2.3 | 2.3 | 2.6 | 2.8 | 2.7 | 2.7 | 3.3 |
| Mean exon length (bp) | 780 | 629 | 624 | 773 | 532 | 446 | 530 | 495 | 477 |
| Introns** | | | | | | | | | |
| Introns (No. introns) | 15,819 | 16,074 | 15,292 | 16,356 | 17,660 | 22,732 | 20,547 | 20,614 | 24,062 |
| Introns/gene | 1.3 | 1.3 | 1.3 | 1.3 | 1.6 | 1.8 | 1.7 | 1.7 | 2.3 |
| Mean intron length (bp) | 73 | 83 | 80 | 91 | 133 | 103 | 114 | 90 | 82 |

*Genomes are *Cercospora beticola* (Cbe), *Cercospora berteroae* (Cbr), *Cercospora canescens* (Ccn), *Dothistroma septosporum* (Dse), *Zymoseptoria tritici* (Ztr), *Leptosphaeria maculans* (Lma), *Pyrenophora tritici-repentis* (Ptr), *Parastagonospora nodorum* (Pno), and *Aspergillus nidulans* (Ani).

**Only considering the longest transcript (if alternatives exist).

*** Not determined (ND).

### 1.6.2. Proteome characterization of *C. beticola* and related fungi

We used BLAST and InterProScan to obtain functional annotation for the full *C. beticola* proteome encompassing in total 12,495 proteins. BLASTp analyses of all *C. beticola* proteins against the NCBI non-redundant database identified significant similarity (E-value ≤ 1e-10; percentage coverage of query ≥ 70%) for 9,580 proteins (77%) whereas similar analyses against a database containing all fungal protein sequences available at the Joint Genome Institute MycoCosm portal resulted in identification of 10,529 proteins (84%) with significant similarity. Similar results were obtained for the six other ascomycete species, ranging from 63% (*L. maculans*) to 86% (*A. nidulans*). InterProScan led to the identification of 12,965 Pfam domains, 5,039 SMART domains and 12,541 superfamily domains in the *C. beticola* proteome. Integration of the BLASTp and InterProScan results by BLAST2GO yielded gene ontology (GO) terms for 7,380 *C. beticola* proteins (59%).

To compare the proteomes of these seven ascomycete fungi including *C. beticola*, all proteins were clustered into 12,167 orthologous protein families based on all-versus-all BLASTp similarity via orthoMCL analysis (Li et al., 2003). From the resulting 12,167 ortholog clusters, we selected 3,554 that contain one protein sequence from each genome. We further filtered these protein clusters by retaining only those that contain single-copy homologs in 39 ascomycete fungi using data from orthoDB (Waterhouse et al., 2012) for phylogenetic analyses, resulting in a reduced set of 850 protein families. Concatenated protein alignments were then used to build a well-supported phylogenetic tree that clusters *C. beticola* most closely with *D. septosporum* and secondly to *Z. tritici* (Fig. 1.3A). This phylogeny is in congruence with the majority of other studies using single- and multi-locus analyses that place the genus *Zymoseptoria* adjacent to the *Cercospora*, *Pseudocercospora*, *Dothistroma* and *Sphaerulina* genera (Arango Isaza et al., 2016; Chang et al., 2016; de Jonge et al., 2018; De Wit et al., 2012; Goodwin et al., 2001; Ohm et al.,

2012). Orthologous protein information was also used to further enhance the functional annotation of the *C. beticola* proteins. More specifically, gene ontology (GO)-terms from orthologous *A. nidulans* proteins, for which extensive GO-term annotation is available through AMIGO (Carbon et al., 2009), were replicated to *C. beticola* proteins. Using this approach, we were able to assign a GO-term to a combined total of 8,608 *C. beticola* proteins.

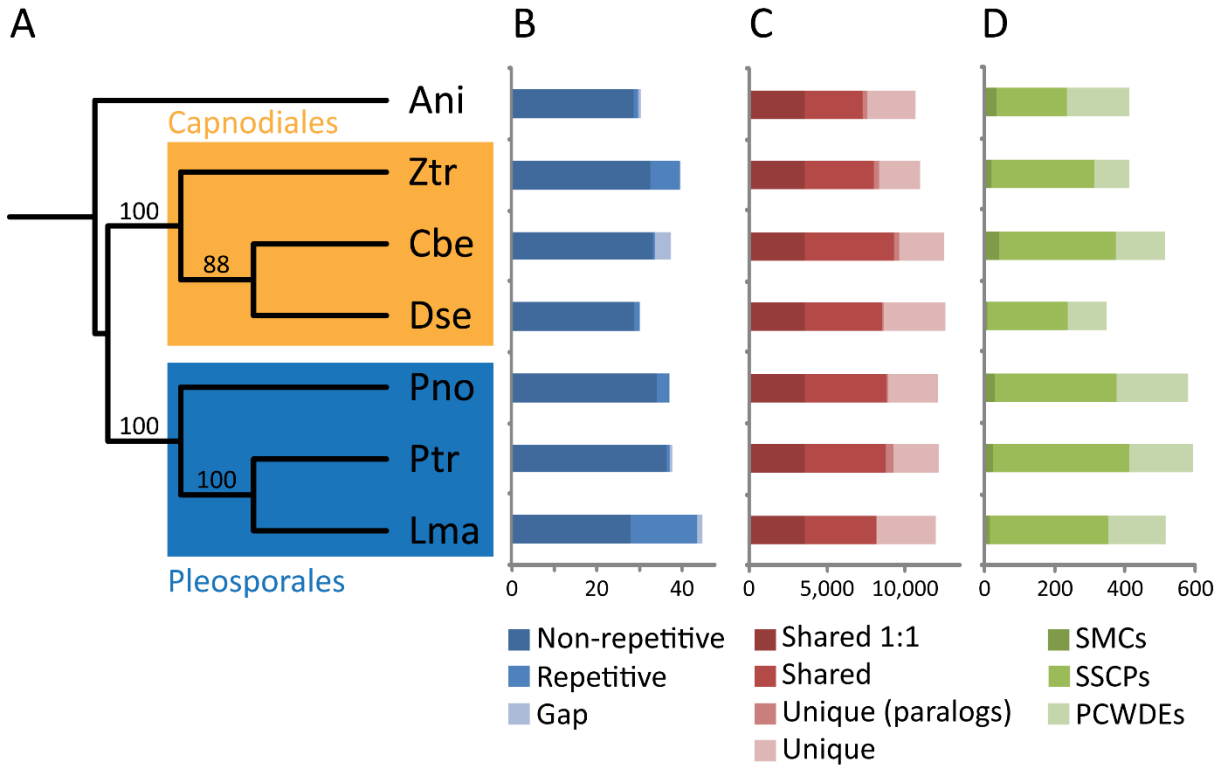



Figure 1.3. Phylogeny and genome characteristics of *Cercospora beticola* and six related fungi. A) Genome-based phylogenetic tree of the six Dothideomycetes, *Zymoseptoria tritici* (Ztr), *Cercospora beticola* (Cbe), *Dothistroma septosporum* (Dse), *Parastagonospora nodorum* (Pno), *Pyrenophora tritici-repentis* (Ptr) and *Leptosphaeria maculans* (Lma), and one Eurotiomycete, *Aspergillus nidulans* (Ani), computed using 850 conserved, single-copy protein families. Bootstrap values are indicated on the branches. B) Genome size and repeat content. C) Number of predicted protein-coding genes, broken down by their appearance (or absence) in orthologous single-copy protein families (Shared 1:1), protein families (Shared), within-species protein families (Unique (paralogs)) or absence from protein families (Unique). D) Number of protein-coding genes that are implicated in plant pathogenesis, broken down by those found in secondary metabolite clusters (SMCs), small, secreted cysteine-rich proteins (SSCPs) and plant cell-wall degrading enzymes (PCWDEs).

### 1.6.3. Secondary metabolite cluster expansion in *C. beticola*

Secondary metabolites (SMs) are small bioactive molecules produced by many organisms including bacteria, plants and fungi (Stringlis et al., 2018). They are abundant in filamentous fungi in which they play crucial roles in the establishment of specific ecological niches. Unlike primary metabolites, SMs are not essential for fungal growth, development or reproduction but contribute to adaptation (e.g. protection against environmental stresses) or pathogenicity. Many enzymes are involved in the synthesis of a single SM. Polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs), which catalyze the elongation of polyketides and oligopeptides, respectively, are among the most prominent and well-studied SM biosynthetic genes. *C. beticola* is known to produce a number of SMs, including cercosporin (Daub and Ehrenshaft, 2000; de Jonge et al., 2018) and beticolin (Goudet et al., 1998). The genome of *C. beticola* possesses 15 type I PKSs, 23 NRPSs, three PKS-NRPS hybrids, six terpene cyclases and one demethylallyl tryptophan synthases (de Jonge et al., 2018). The closely related Dothideomycetes *D. septosporum* and *Z. tritici* possess a significantly lower number of both type I PKSs (3 and 10, respectively) and NRPSs (5 and 8, respectively). The expansion of NRPSs in *C. beticola* is particularly notable when compared to *L. maculans, P. nodorum* and *P. tritici-repentis* (this study) and *C. fulvum* (De Wit et al., 2012). The expansion in NRPSs is further exemplified by a high number of NRPS-related Pfam domains (PF00501, PF00550, PF00668 and PF13193). Interestingly, the predicted *C. beticola* SM clusters are preferentially found in sub-telomeric regions (Fig. 1.4A), a common feature of fungal SM clusters (Palmer and Keller, 2010). Likely, the observed co-regulation of expression among genes within SM clusters is achieved by epigenetic forces such as chromatin-level control (Palmer and Keller, 2010). Chromosomal location of gene clusters near centromeres and telomeres possibly correlates with

regions that experience facultative heterochromatin, i.e. large regions that can efficiently be silenced as well as activated by chromatin-mediating factors (Palmer and Keller, 2010).

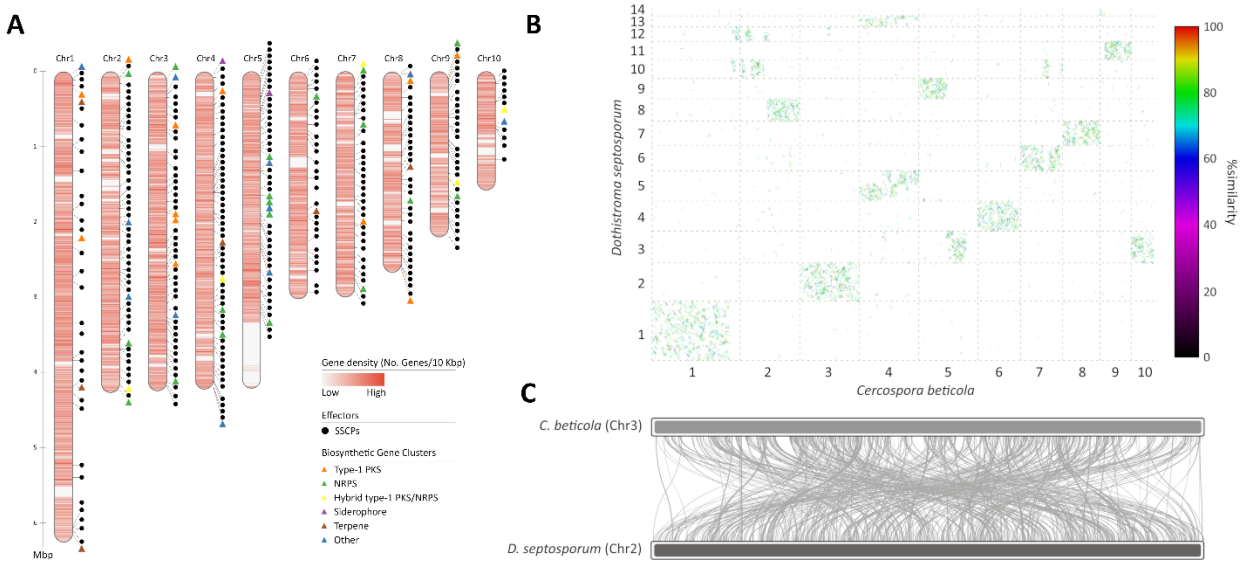



Figure 1.4. The *Cercospora beticola* genome and whole-genome comparisons with *Dothiostroma septosporum*. A) Schematic representation of the 10 largest (pseudo)chromosomes and/or scaffolds of *C. beticola*, highlighting the density of genes (genes/10 Kbp; ranging from 0 (white) to 10 (dark red)), the location of candidate effectors (the small, secreted cysteine-rich proteins or SSCPs; black circles) and the type and location of biosynthetic gene clusters (colored triangles) for secondary metabolites. B) Whole-genome alignment between *Cercospora beticola* and *Dothistroma septosporum* highlighting extensive chromosome mesosynteny. Whole-genome, protein sequence-based alignments between *C. beticola* and *D. septosporum* reveal many short syntenic regions that are spread over each scaffold or chromosome pair, exemplary for mesosynteny. C) Example of the shuffled homologous regions shared between chromosomes 3 and 2 from *C. beticola* and *D. septosporum*, respectively. A) and C) prepared using RIdeogram (Hao et al., 2019) while B) prepared by *promer* in MUMMER3 (Kurtz et al., 2004).

### 1.6.4. Definition and annotation of the *C. beticola* secretome

The arsenal of potentially secreted proteins (i.e. the secretome) among plant pathogens includes key pathogenicity molecules that are generally referred to as effectors (Kamoun, 2007). To identify candidate effectors, we scanned the proteome of *C. beticola* for proteins that are predicted to be secreted and lack transmembrane domains. We identified 1,087 such proteins of which 333 exhibited additional effector characteristics including small size (≤300 amino acids) and cysteine-rich (≥2 cysteine residues) (Fig. 1.4A; SSCPs). Analysis of genomic localization

revealed an underrepresentation of SSCPs on Chromosome 1 ($p < 0.0001$; Chi-square test; Fig. 1.4A) that might be an indication of genome compartmentalization, often referred to as the two-speed genome (Raffaele et al., 2010). Of the 333 candidate effectors, 62 (approximately 19%) had no BLASTp hits in the non-redundant database from NCBI nor in the collection of 347 fungal proteomes obtained from the MycoCosm fungal genomics portal and are therefore considered *C. beticola*-specific.

### 1.6.5. *C. beticola* synteny with related fungi

Conservation of sequence between chromosomes or scaffolds of different species or strains is typically referred to as synteny. Through gene loss, inversions, translocations or other chromosomal rearrangements, synteny between homologous chromosomes (i.e. of common descent) can be broken. Major chromosomal rearrangements are, however, rare when comparing distantly related eukaryotes, and as a result, large collinear regions with similar genes in similar order and orientation can be found across these species. This kind of 'global' chromosome conservation is known as macrosynteny. Such macrosynteny is only rarely observed among species in the fungal kingdom. In fungi, a type of synteny known as microsynteny is common in which small segments of up to 10 conserved genes can be found. Moreover, a phenomenon termed mesosynteny in which homologous chromosomes display significant conservation of gene content but not gene order is typical in the ascomycete fungi and especially prominent in the Dothideomycete class to which *C. beticola* belongs (Hane et al., 2011). Conservation of gene content but not of gene order can be attributed to a large number of intra-chromosomal rearrangements such as inversions and a very limited number of inter-chromosomal rearrangements. Hane et al. (2011) hypothesized that a high frequency of inversions might occur during meiosis, although this does not explain why Dothideomycetes in particular, and only

ascomycete fungi, display such a striking conservation of chromosome gene content since many species in this class do not have a known sexual stage.

To investigate whether *C. beticola* chromosomes also display mesosynteny to related fungal species, we aligned *C. beticola* chromosomes with those of the closely related fungus, *D. septosporum* (Fig. 1.4B & C). Notably, strict conservation of chromosome content but not order can be observed, the hallmark for mesosynteny. Considering a limited number of intra-chromosomal rearrangements, it is expected that some collinear segments remain because insufficient rearrangements have occurred to randomize the complete gene order within a chromosome. On the other hand, some collinear segments might be the result of continuing selection pressure to keep particular genes involved in the same or similar biological processes tightly clustered on the genome, as we have previously observed is the case for biosynthetic gene clusters for cercosporin (de Jonge et al., 2018; Ebert et al., 2019) and melanin (Ebert et al., 2019)

## 1.7. *C. beticola* effector repertoire

### 1.7.1. The definition of an effector

Effectors can be described as microbially secreted molecules such as proteins, SMs, and small RNAs that contribute to niche colonization (Rovenich et al., 2014; Snelders et al., 2018). This definition implies that effector functions are not necessarily restricted to interaction with a host plant but also include involvement in microbial competition and nutrition acquisition (Fatima and Senthil-Kumar, 2015; Snelders et al., 2018) which may have roles during a saprophytic growth period. Effectors that are secreted during host colonization may have the ability to modulate host biochemistry and physiology, including defense responses, to facilitate host colonization (de Jonge et al., 2011). The type of effectors produced depends on the lifestyle of the pathogen and stage of infection. Biotrophic fungi obtain their nutrients from living plant tissues and their effectors typically act to inhibit plant defense responses and facilitate nutrient

acquisition. Necrotrophic fungi, however, secrete toxins and proteins to evoke host cell death and induce nutrient release (Koeck et al., 2011). The hemibiotrophic lifestyle of *C. beticola* begins with a latent asymptomatic (biotrophic) phase where invasive hyphae grow within the host plant followed by a fine-tuned transition to necrotrophy. The two different phases of growth likely require different effector repertoires. Future time-course transcriptome studies of sugar beet infection may help to elucidate how phase transition occurs and identify candidate effectors contributing to biotrophy or necrotrophy as has been shown in other pathosystems (Gan et al., 2013; Rudd et al., 2015; Zuluaga et al., 2016). To date, both SM and proteinaceous effectors have been identified in *C. beticola* and will be discussed below.

### 1.7.2. Cercosporin

#### *1.7.2.1. The role of cercosporin*

Cercosporin is a light-activated, non-host specific toxin produced by most *Cercospora* species (Daub and Ehrenshaft, 2000). Mutant lines that are unable to produce this SM experience a virulence penalty, implicating cercosporin as a virulence factor for *C. beticola*, *C. nicotianae*, *C. kikuchii*, *C. coffeicola* and *Cercospora cf. flagellaris* (Callahan et al., 1999; Choquer et al., 2005; Rezende et al., 2019; Santos et al., 2019; Souza et al., 2019; Weiland et al., 2010). However, there are *Cercospora* species that naturally lack the ability to produce this toxin and yet are virulent phytopathogens, demonstrating that cercosporin is not solely necessary for pathogen success in the genus (Swart et al., 2017; Weiland et al., 2010). The metabolic pathway genes involved in cercosporin formation are organized in a cercosporin toxin biosynthesis (CTB) cluster. While cercosporin was thought to be a unique feature of fungi that belong to the *Cercospora* genus, it has been shown that the CTB cluster experienced duplications and multiple horizontal gene transfers across a variety of taxa, including many *Colletotrichum* species of which *C. fioriniae* has been confirmed to also produce this potent toxin (de Jonge et al., 2018).

*1.7.2.2. Toxicology*

The photosensitizing nature of various perylenequinones, including cercosporin, has long been known (Brockmann et al., 1950; Weiss et al., 1957; Yamazaki et al., 1975). The essential structural feature responsible for photodynamic activity is the 3,10-dihydroxy-4,9-perylenequinone chromophore (Hudson et al., 1997), which allows absorption of visible and near-UV light that elevates the perylenequinone to an electronically excited triplet state (Foote, 1976; Foote, 1968). Once in this activated triplet state, two types of reactions are known to occur (DeRosa and Crutchley, 2002; Guedes and Eriksson, 2007); the excited perylenequinone can react with oxygen either indirectly (type I reaction) through a reducing substrate or directly (type II reaction) (Fig. 1.5). Interaction with an electron donor leads to the formation of free radicals or radical ions that in turn react with oxygen to produce reactive oxygen species (ROS) such as $H_2O_2$ and free radical forms such as $O_2^{\bullet-}$, $HO_2^{\bullet}$, $OH^{\bullet}$. In a direct interaction between a triplet perylenequinone and oxygen, energy can be transferred from the excited triplet state perylenequinone to oxygen resulting in an excited singlet state of oxygen, also known as “singlet oxygen” ($^1O_2$). Both type I and II reactions yield ROS that at high concentrations are harmful to cells as they can cause lipid peroxidation as well as protein and DNA damage (Birben et al., 2012; Blokhina et al., 2003).

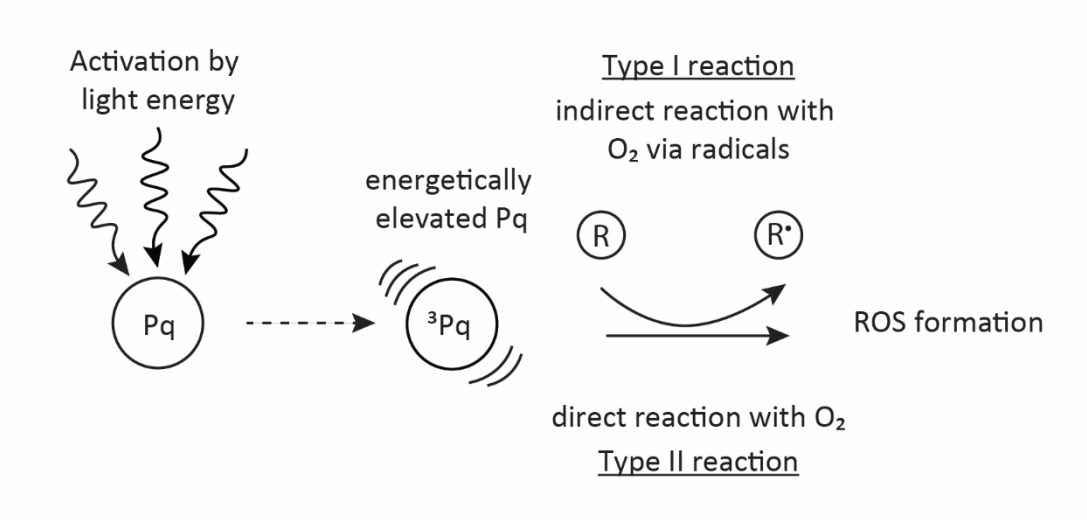



Figure 1.5. Perylenequinone mode-of-action. Light exposure activates perylenequinones (Pq) to reach an energetically excited triplet state ($^3$Pq) in which they can react with oxygen ($O_2$) to form reactive oxygen species (ROS). This reaction can happen indirectly (type I reaction) where the activated perylenequinone reacts with a reducing substrate (R) first resulting in radical formation ($R^{\bullet}$ and $Pq^{\bullet-}$) that can react with $O_2$ to form ROS. Alternatively, the activated perylenequinone can react directly with $O_2$ (type II reaction) to form ROS.

*1.7.2.3. Biosynthesis*

While extensive research on the CTB pathway has shed light on selected pathway steps, it has not yet been possible to determine a full biosynthesis scheme due to extreme instability of most pathway intermediates and the potential occurrence of feedback inhibition (Newman and Townsend, 2016). To date, 12 genes have been found to be part of the CTB biosynthetic pathway, either involved in cercosporin production or export (Fig. 1.6A). The iterative, nonreducing PKS CTB1 is essential for cercosporin production and acts as the keystone enzyme to initiate the biosynthetic process (Choquer et al., 2005; Crawford and Townsend, 2010; Newman et al., 2012). CTB1 harbors six functional domains that conjointly work together to form *nor*-toralactone, the first intermediate in the cercosporin assembly line using one acetyl-CoA and 6× malonyl-CoA units as substrate (Choquer et al., 2005; Newman and Townsend, 2016; Newman et al., 2012). Next, the *nor*-toralactone intermediate is processed to toralacatone and subsequently to cercoquinone C by CTB3, a predicted *O*-methyltransferase FAD-dependent

monooxygenase (de Jonge et al., 2018; Dekkers et al., 2007; Newman and Townsend, 2016). Further processing of this intermediate may be mediated by CTB2, CTB6, and CTB11/CTB12, which are hypothesized to methylate, reduce and dimerize the molecule, respectively (Newman and Townsend, 2016; Staerkel et al., 2013). However, the corresponding intermediates have not been directly observed but are rather logically inferred. The CTB5 and/or CTB7 gene products are hypothesized to prime the cercosporin intermediate for methylenedioxy bridge formation by CTB9 and CTB10 to yield the final cercosporin molecule (Chen et al., 2007; de Jonge et al., 2018; Newman and Townsend, 2016; Swart et al., 2017). The CTB8 gene product is a $Zn(II)Cys_6$ zinc finger transcription factor and not directly involved in the modification of the toxin itself but is responsible for mediation of the CTB gene cluster expression (Chen et al., 2007). Lastly, the gene cluster holds two major facilitator superfamily (MFS) transmembrane transporters, CTB4 and the cercosporin facilitator protein (CFP) (Chen et al., 2007; Choquer et al., 2007; de Jonge et al., 2018). *C. nicotianae* CTB4 mutant strains displayed a reduction in cercosporin production by at least 35% while cercosporin accumulated in fungal mycelium and was not secreted into the medium, suggesting that CTB4 mediates cercosporin export (Choquer et al., 2007). When stimulated by high light conditions, *CTB4* mutants secreted a dark brown compound of unknown nature that quickly diffused into the solid medium. Interestingly, a *CTB4* homolog is also present in the putative phleichrome biosynthetic pathway, but is lacking in the predicted elsinochrome and hypocrellin clusters (Ebert et al., 2019). As elsinochrome and hypocrellin are secreted by *E. fawcettii* and *S. bambusicola*, respectively, despite the lack of a *CTB4* homolog, the question arises whether CTB4 is indeed solely responsible for toxin export or whether other transporter proteins can functionally substitute toxin export in the absence of CTB4 (Choquer et al., 2007). The *CFP* gene is incorporated in the CTB cluster of *C. beticola* (de

Jonge et al., 2018) and is hypothesized to partially provide tolerance to cercosporin (auto-resistance) via toxin export (Callahan et al., 1999).

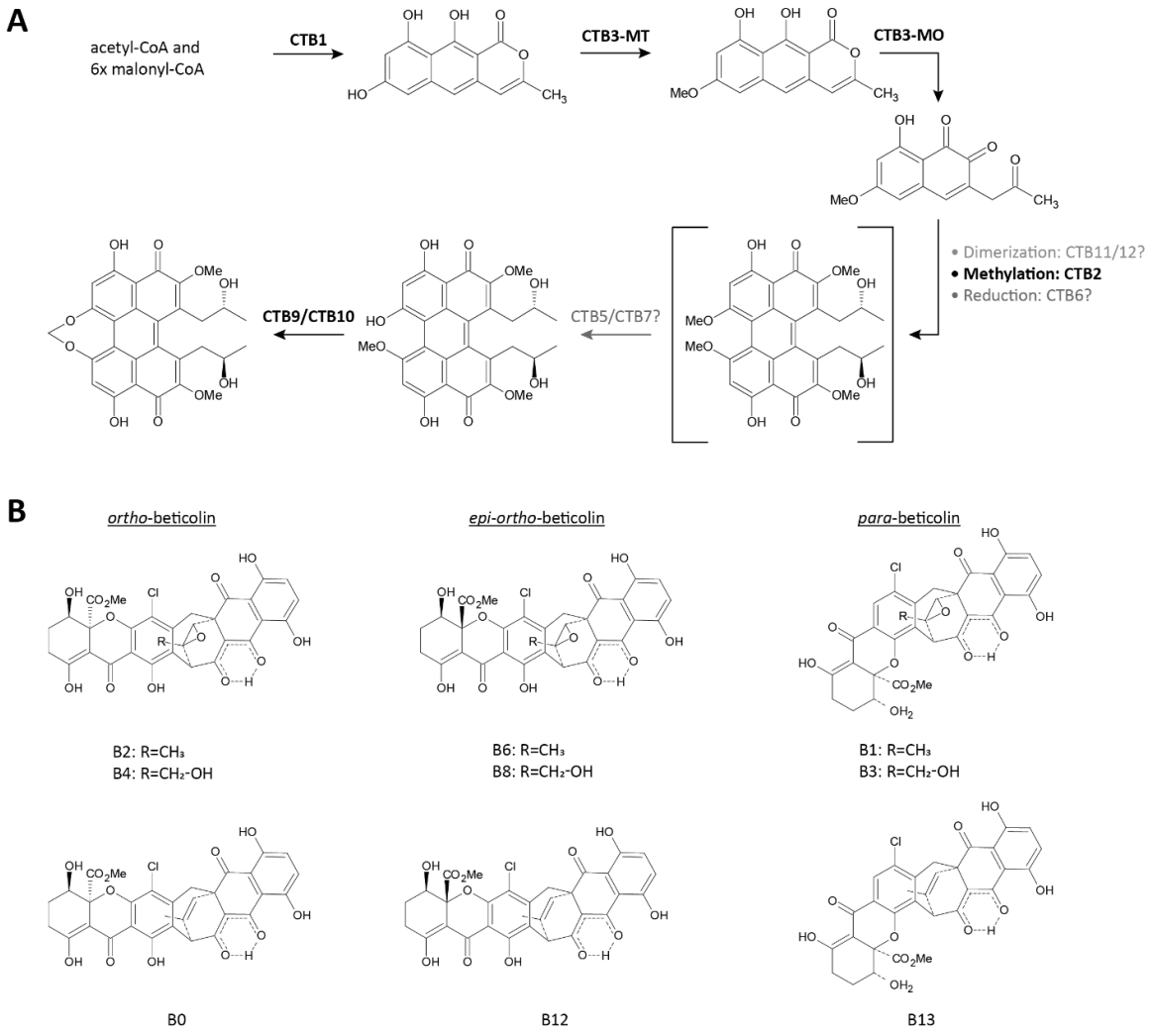



Figure 1.6. The cercosporin biosynthetic pathway and beticolin isoforms. A) A preliminary scheme of the cerocosporin biosynthetic pathway consisting of 12 clustered genes. The polyketide synthase CTB1 forms nor-toralactone that is processed to cercoquinone C by CTB3 methyltransferase (CTB3-MT) and monooxygenase (CTB3-MO). Further processing of this cercosporin intermediate might by mediated by CTB2, CTB6, and CTB11 and CTB12 to yield the cercosporin intermediate displayed in the square bracket which has not been directly observed but is rather logically inferred. CTB5 and CTB7 are hypothesized to prime the cercosporin molecule for methylenedioxy bridge formation by CTB9 and CTB10. B) Beticolin structures and isoforms. Beticolins are structurally related but can differ by residues (R) and isoforms (*ortho*-, *epi-ortho*-, or *para*-beticolin). Beticolins that carry the same residues are able to transform into each other by switching isomery. For example, *ortho*-beticolin B2 can transform into the *epi-ortho*-beticolin B6 or *para*-beticolin B1 based on the position of the oxygen and chlorine atoms.

*1.7.2.4. Auto-resistance*

It is essential for fungi that produce antifungal SMs to protect themselves from their own toxin. The phenomenon of avoiding self-intoxication is known as auto-resistance (AR). Genes with potential AR roles have been identified in highly conserved toxin biosynthesis gene clusters, with no direct involvement in toxin production. Three self-resistance strategies have been highlighted in the literature: toxin export, detoxification, and duplication of the toxin target (Keller, 2015). However, to this date only toxin export via transporter proteins and detoxification of toxic compounds have been found in cercosporin-producing fungi.

Protection by toxin efflux relies on the transportation of toxic substances from the inside of a cell to the outside through membrane transport proteins. Transporters involved in self-resistance mainly belong to the MFS and ABC superfamilies (Cannon et al., 2009; Costa et al., 2014). The MFS transporter protein, CFP, was first identified in *C. kikuchii* and shown to be involved in cercosporin AR as targeted gene disruption of *CFP* resulted in mutant strains with increased sensitivity to exogenous cercosporin (Callahan et al., 1999). With the recent discovery that the CTB cluster consists of additional genes (de Jonge et al., 2018), it was possible to demonstrate that the MFS transporter homolog in *C. beticola*, *CbCFP*, is incorporated in the cluster and flanked on both sides by genes necessary for cercosporin production (de Jonge et al., 2018). Mutants that lack *CbCFP* are more sensitive to exogenous cercosporin *in vitro* (Ebert and Bolton, *unpublished data*), suggesting that this AR mechanism is conserved in the *Cercospora* genus.

Besides toxin export, *Cercospora* species are known to have a second mechanism of AR through reductive detoxification of the cercosporin molecule (Daub et al., 1992; Daub et al., 2000; Leisman and Daub, 1992; Sollod et al., 1992). It has been shown that stably methylated and acetylated reduced cercosporin derivatives absorb less light and generate significantly less

singlet oxygen ($^1O_2$) compared to wild-type cercosporin (Leisman and Daub, 1992). Further investigation has revealed that the cell surface of cercosporin-resistant strains is surrounded by a reducing environment (Leisman and Daub, 1992), showing that resistant fungi can reduce proximate cercosporin into its less reactive form and maintain this detoxification so long as the molecule is nearby. Although specific genes have been reported in other fungi that are linked to active cercosporin detoxification (Panagiotis et al., 2007; Ververidis et al., 2001) no CTB genes have yet been affiliated with AR via cercosporin reduction.

### 1.7.3. Beticolins

Beticolins are a group of non-host specific phytotoxins of which 20 members (B0 to B19) have been identified to be produced by *C. beticola* (Goudet et al., 2000; Goudet et al., 1998; Milat and Blein, 1995) and the hoary alyssum (*Berteroa incana*) pathogen *C. berteroae* (Assante et al., 1977). Alternative names for these toxins, such as Gelbe Fration (Langfelder et al., 2003; Schlösser, 1962), *Cercospora beticola* toxin (CBT) (Assante et al., 1977), and cebetins (Jalal et al., 1992; Robeson and Jalal, 1993) arose due to simultaneous research efforts by different groups and limited data concerning their structure during early research. Later, analyses of their chemical structures revealed that beticolins are structurally closely related (Fig. 1.6B). All have a chlorine atom attached to the central aromatic ring, while their octocyclic basic structure is composed of two subunits; a partially hydrogenated anthraquinone and a partially hydrogenated xanthone that are connected through a seven-membered ring (Ducrot et al., 1996; Ducrot et al., 1994; Goudet et al., 2000; Goudet et al., 1998; Simon-Plas et al., 1996). Structural differences between beticolins are due to different isomeric configurations (*ortho-*, *para-*, or *epi-ortho-*) and by variable residues (Milat et al., 2010). Interestingly, beticolins can switch isomery. For example, *ortho*-beticolin B2 is able to transform into the *para*-beticolin B1 or *epi-ortho*-beticolin B6 and vice versa (Ducrot et al., 1994). Early research on their biological function indicated that

beticolins have antibacterial and phytotoxic properties (Schlösser, 1962). However, necrosis formation in plants upon beticolin application was only induced in the presence of light. Later it was found that due to their ability to form complexes with $Mg^{2+}$, beticolins inhibit tumoral cell growth in mice (Ding et al., 1996; Ding et al., 2001), interfere with $H^{+}$-ATPase activity (Gomès et al., 1996a; Gomès et al., 1996b; Gomès et al., 1996c; Simon-Plas et al., 1996) and are able to incorporate themselves into lipid bilayers to form ion channels with poor ion selectivity (Goudet et al., 1999; Goudet et al., 2000; Goudet et al., 1998). The latter property led to the classification of beticolins as ion channel-forming toxins (Goudet et al., 2000). While chemical structures and biological activity have been evaluated throughout the last decades, the biosynthetic pathway of these toxins is unknown. Therefore, it is currently not possible to assess to what extent beticolin production and associated phytotoxic effects contribute to *C. beticola* virulence.

### 1.7.4. Melanin

Fungal phytopathogen melanin production has been implicated in appressorial penetration of host plants and pathogenesis as well as being an integral component of the cell wall that can be useful for tolerating environmental stresses (Langfelder et al., 2003; Liu and Nizet, 2009). Recent research mining the *C. beticola* genome for novel PKS genes has revealed the secondary metabolite production of melanin (Ebert et al., 2019). Phylogenetic analysis of the *CbPKS1* gene belonging to the DHN-melanin clade grouped *C. beticola* with other well-characterized melanin biosynthetic clusters from various ascomycetes. Additionally, whole-cluster homology with other PKS genes that are involved in melanin production gave further evidence that these genes may be functional. Knock-out mutants in melanin *CbPKS1* resulted in albino phenotypes that did not affect the PKS biosynthetic cluster involved in cercosporin production (Ebert et al., 2019). Interestingly, a gene adjacent to *CbPKS1* encoding for a predicted tetra-hydroxynaphthalene reductase (*Cb4HNR*) involved with melanin biosynthesis has

been shown to be induced in fungicide-resistant *C. beticola* strains (Bolton et al., 2016). This proposes a role for the SM production of melanin that is likely an adaptation involved in survival in a challenging environment.

### 1.7.5. Protein effectors

Effector proteins are known to be employed by a broad variety of plant pathogenic fungi to evade detection by the host during colonization. Upon pathogen recognition, the plant will initiate defense responses such as the production of chitinases that target fungal hyphae for degradation. To shield the fungal cell wall from plant-derived chitinases, the biotrophic fungus *C. fulvum* that causes leaf mold on tomato secretes the virulence factor CfAvr4 (van Esse et al., 2007). This effector can bind to chitin in the fungal cell wall and protect fungal hyphae from hydrolysis by plant chitinases (Chang and Stergiopoulos, 2015; van den Burg et al., 2004; van den Burg et al., 2003; van Esse et al., 2007). A homologous gene was discovered in *C. beticola* (Stergiopoulos et al., 2010) and *Cercospora* cf. *flagellaris* (Rezende et al., 2019) and *in vitro* studies found that the *CbAvr4* and *Cfla-Avr4* gene products could functionally bind to chitin (Mesarich et al., 2016; Rezende et al., 2019). As chitin-binding appears to be a conserved biological trait between CfAvr4 and Avr4 homologs, it is hypothesized that Avr4 homologs including CbAvr4 also share the CfAvr4 function of shielding fungal hyphae from lysis by plant chitinases.

Effector protein identification through comparative genomics has served as a useful tool to detect another *C. beticola* effector named CbAve1. CbAve1 is a homolog of VdAve1 (Avirulence on Ve1 tomato), an effector secreted by the vascular wilt pathogen *Verticillium dahliae*. In *V. dahliae* the VdAve1 effector is recognized by the tomato cell surface-localized immune receptor Ve1 and confers resistance to the pathogen. In the absence of Ve1, VdAve1 has been shown to contribute to fungal virulence but the mechanism remains elusive (de Jonge et al.,

2012). In the *C. beticola-B. vulgaris* system, *CbAve1* is expressed during infection and its product acts as a virulence factor (Boshoven et al., 2015)**.**

Due to its hemibiotrophic lifestyle, it was hypothesized that *C. beticola* also secretes proteinaceous effectors that promote its necrotrophic phase. Using a phenotype-based forward genetics approach, a proteinaceous virulence factor named CbNIP1 (*Cercospora beticola* necrosis-inducing protein 1) was identified due to its ability to necrotize sugar beet and *Nicotiana benthamiana* leaves (Ebert et al., 2018). Interestingly, CbNIP1's ability to induce necrosis within 48 hours was highly regulated by light. While other necrosis-inducing proteins such as ZtNIP1 and ZtNIP2 of the wheat pathogen *Z. tritici* require light for full functionality (M'Barek et al., 2015), CbNIP1 was most active in complete darkness as exposure of CbNIP1-infiltrated sugar beet leaves with a 12 hour light-dark cycle led initially to chlorosis formation that gradually turned necrotic over time (Ebert et al., 2018). Furthermore, CbNIP1 appears to contribute to necrotic symptom development, as upregulated *CbNIP1* expression *in planta* correlates with necrotic lesion appearance. At present, the mode of action of CbNIP1 and its location during infection remains unknown.

## 1.8. Disease management

### 1.8.1. Integrated practices

The integrated management of CLS is comprised of cultural practices, moderate host resistance and the timely application of fungicides. Cultural practices aim to reduce the amount of initial inoculum for the following season through rotation with non-host crops, tillage (burying infested debris) and avoiding planting next to fields previously sown with sugar beets. Epidemiological models regarding the disease progress of CLS have been established to predict disease severity and timing of fungicide application (Pitblado and Nichols, 2005; Racca and Jörg, 2007; Rossi and Battilani, 1991; Windels et al., 1998). Fungicides should be sprayed early

in a protective manner to avoid the development of conidial populations which can infect new unprotected foliage. Although there have been several studies regarding the potential of different bacteria and fungi as biocontrol agents for CLS, including *Trichoderma* and *Bacillus subtilis* (Collins and Jacobsen, 2003; Galletti et al., 2008), there have been no current reports of their success as a management tool in the field. Alternatively, the presence of several microbial groups has been correlated with disease incidence in sugar beet fields and these microbes may be useful as biological markers for predicting disease outbreaks (Kusstatscher et al., 2019).

### 1.8.2. Host resistance

#### *1.8.2.1. Genetic improvement of CLS resistance in sugar beet*

The improvement of CLS resistance in sugar beet varieties over the last few decades has been a concerted effort by geneticists and breeders. Wild sea beet, *B. vulgaris* L. spp. *maritima*, has long been a source of CLS resistance genes (Rossi, 1995). Other wild *Beta* relatives, such as *B. procumbens*, have displayed non-host resistance but are sexually incompatible with *B. vulgaris* (Panella and Frese, 2000). Inheritance of CLS resistance in sugar beet lines after introgression from wild sea beet is complex and has low heritability (Smith and Ruppel, 1974) while at the same time, incorporation into high-yielding commercial sugar beet hybrids remains a challenge (Smith and Campbell, 1996). Promisingly, recent field trials in Germany showed that several European sugar beet varieties with CLS resistance lacked a yield penalty in the absence of disease and had better economic performance than susceptible varieties (Vogel et al., 2018). CLS resistance is typically managed by at least four identifiable QTLs (Nilsson et al., 1999; Setiawan et al., 2000; Smith and Gaskill, 1970; Taguchi et al., 2011). Precise mapping of resistance QTLs helps marker-assisted selection (MAS) in breeding programs to introgress CLS resistance (Taguchi et al., 2011). The more precise the mapping, the higher the chances of breaking the potential linkage between CLS resistance and unfavorable traits. The underlying

gene products can also be identified and used as molecular markers to identify alleles associated with resistance (Hunger et al., 2003). Previously, monogenic resistance was identified to race C2 of *C. beticola* in a sugar beet cultivar (Lewellen and Whitney, 1976) but the resistance was shown to be unstable (Koch and Jung, 2000). No other examples of monogenic resistance to CLS have been described since in sugar beet. Current and future breeding efforts for CLS resistance can exploit the reference-quality 567 Mb genome sequence of sugar beet with 27,421 transcript-supported genes published by Dohm et al. (2014). An additional reference-quality genome was recently developed for a different sugar beet variety with focused annotation of nucleotide-binding (NB-ARC), leucine-rich repeat (NLR) disease resistance genes, including 231 tentative NB-ARC loci (Funk et al., 2018). When comparing these loci to validated resistance genes from monocots and eudicots, there appeared to be extensive *B. vulgaris*-specific subfamily expansions. Draft genomes of *B. patula* and sea beet were also recently released (del Río et al., 2019) and represent valuable resources for sugar beet breeding research.

*1.8.2.2. Molecular basis of host defense*

Although few studies have focused on the molecular basis of resistance, there is some published work examining plant defenses upon *C. beticola* infection. The interaction between sugar beet and *C. beticola* begins with an initial defense response by the plant upregulating phenylalanine ammonia lyase (PAL) involved in the various biosynthetic pathways for many plant-related SM compounds (i.e. lignins, flavonoids and phytoalexins) utilized during biotic attacks (Liang et al., 1989). These plant defense mechanisms have been found to be suppressed by *C. beticola* via an interaction with a pathogen-induced molecule on the PAL core promoter (Schmidt et al., 2004). Later it was discovered that upon initial infection with the pathogen and through mid- to late-stages of disease development, the plant hormone abscisic acid (ABA) was elevated (Schmidt et al., 2008). ABA was also found to reduce PAL gene expression in sugar

beet through an unknown mechanism (Schmidt et al., 2008). ABA has been found to interfere with biotic stress signaling in other plants, including via suppression of PAL transcription and activity, which negatively impacts disease resistance (Mauch-Mani and Mauch, 2005). Additional research into the defense response of sugar beet cultivars to CLS showed that three cultivars with varying resistance genotypes (susceptible, polygenic partial resistance or monogenic resistance) differed in timing and strength of defense reactions upon infection (Weltmeier et al., 2011). In all three cultivars, genes were activated in hormone production (ethylene, jasmonic acid and gibberellin), lignin and alkaloid synthesis, signaling and pathogenesis-related (PR) genes by the time symptoms had appeared. The monogenic resistant genotype (resistant to *C. beticola* isolate C2) displayed strong defenses (*PR* and *WRKY* gene expression) 1-day post-inoculation and there was no significant increase in *C. beticola* biomass. The partial resistance genotype had a stronger defense response than the susceptible genotype and a 50% reduction in *C. beticola* biomass, but the pathogen was still able to infect and cause disease when there was late initiation of defense responses at 15-days post-inoculation.

### 1.8.3. Fungicide usage and resistance development

#### *1.8.3.1. Fungicide classes employed for CLS management in sugar beet*

The use of fungicides has been an integral part of CLS management primarily due to the lack of effective non-chemical alternatives. There are two main types of chemistries available for disease management: protectant fungicides with broad spectrum activity and systemic fungicides that target a specific site in the fungus. Of the former, the most commonly used are the ethylene bisdithiocarbamate (EBDC, Fungicide Resistance Action Committee or FRAC Group M03) class of fungicides, copper-based fungicides (FRAC group M01) and, in the US, the organotin class of compounds (FRAC Group 30) such as triphenyltin hydroxide and triphenyltin acetate. The three main classes of systemic fungicides that have been employed globally are the benzimidazoles

(FRAC Group 1) and triazoles (sterol demethylation inhibitors or DMIs, FRAC Group 3), and quinone outside inhibitors (QoIs, FRAC Group 11).

The continued efficacy of these fungicide classes has been marred by the emergence of resistant strains in *C. beticola* populations over the last few decades (Fig. 1.7). *C. beticola* resistance has been noted to occur after widespread and repeated use of the same fungicide classes (Giannopolitis, 1978; Rosenzweig et al., 2020; Secor et al., 2010). Other factors contributing to the development of fungicide resistance are the polycyclic nature of this pathogen, its high rate of sporulation and common spray programs being used over large areas for disease management (Dekker, 1986). The rotated use of different fungicide classes has been implemented to suppress selection for fungicide-resistant *C. beticola* strains. Systemic fungicides (such as DMIs) are also commonly mixed with a protectant fungicide for higher efficacy, reduced costs and as an additional step in resistance management (Ioannidis, 1994).

With the emergence of resistance to most available fungicide chemistries, many recent studies have focused on characterizing the molecular basis of resistance. For fungicides with single target sites, such as the benzimidazoles, QoIs and DMIs, target genes can be sequenced and compared in both sensitive and resistant isolates to identify likely causal mutations.

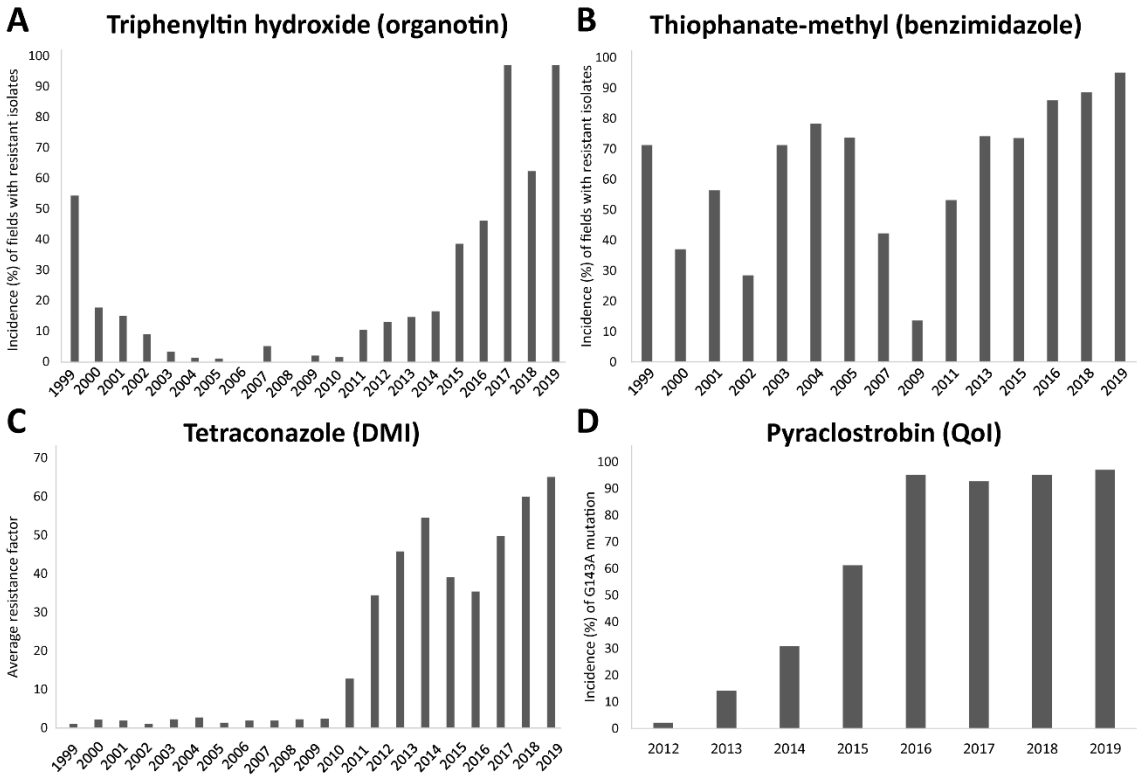



Figure 1.7. Fungicide resistance surveys for *Cercospora beticola* in the Red River Valley region. A) Incidence (percentage) of fields sampled annually from 1999-2019 with isolates resistant to the organotin fungicide triphenyltin hydroxide at 1 µg/mL. B) Incidence (percentage) of fields sampled from 1999-2019 with resistance to the benzimidazole fungicide thiophanate-methyl at 5 µg/mL. C) Average annual resistance factor values to the demethylation inhibitor (DMI) fungicide tetraconazole for isolates sampled annually from 1999-2019, where the resistance factor values are the calculated $EC_{50}$ values divided by the baseline sensitivity values. D) Incidence (percentage) of sampled isolates annually from 2012-2019 harboring the G143A mutation in cytochrome b, conferring resistance to the quinone outside inhibitor (QoI) fungicide pyraclostrobin.

*1.8.3.2. FRAC Group 30*

Triphenyltin acetate was used in Europe in the 1970s, whilst triphenyltin hydroxide was used extensively in the U.S. throughout the 1980s (Windels et al., 1998). Tolerance to triphenyltin fungicides quickly emerged in Greece (Giannopolitis, 1978), Serbia (Marić et al., 1984), North Dakota and Minnesota (Bugbee, 1995; Campbell et al., 1998). However, it was noted that triphenyltin-resistant strains of *C. beticola* were less competitive than sensitive strains

in the absence of this fungicide (Giannopolitis and Chrysayi-Tokousbalides, 1980). Furthermore, annual surveys of triphenyltin hydroxide resistance in *C. beticola* isolates from central North America's Red River Valley (RRV) region have also suggested that a fitness penalty is associated with triphenyltin hydroxide resistance (Secor et al., 2010). Recent surveys of this region have shown high incidence of triphenyltin resistance (97% of isolates surveyed in 2017) but the severity of resistance was low (average spore germination rate was still <30%), perhaps suggesting that organotins may still be effective fungicides (Secor et al., 2017). Organotins act by inhibiting ATP synthase activity to stop oxidative phosphorylation in mitochondria but the genetic basis of resistance in fungi is unknown (Gadd, 2000). Although one of the most effective fungicide groups in use, organotins are no longer permitted for use within the European Union because of their associated consumer risks (Risk & Policy Analysts Limited, 2005).

*1.8.3.3. FRAC Group 1*

Benzimidazole fungicides were implemented in the early 1970s. The first report of economic losses due to benzimidazole resistance in *C. beticola* populations was in Greece in 1973 (Georgopoulos and Dovas, 1973), followed by appearances in other production areas worldwide such as the U.S. (Bugbee, 1982; Ruppel and Scott, 1974), China (Dafang and Shuzhi, 1982) and India (Pal and Mukhopadhyay, 1983). EBDC and DMI fungicides were subsequently introduced to manage these resistant populations alongside the organotins. Benzimidazoles inhibit microtubule assembly during mitosis by binding to β-tubulin subunits (Davidse, 1986). Sequencing of the target β-tubulin gene identified a glutamic acid to alanine amino acid change at codon 198 (designated E198A) associated with high benzimidazole resistance in multiple populations of *C. beticola* in the U.S. and Europe (Davidson et al., 2006; Trkulja et al., 2013). An additional β-tubulin mutation, phenylalanine to tyrosine at codon 167 (designated F167Y) has been found in low-to-moderate resistant isolates from Serbia (Trkulja et al., 2013), causing

F167Y isolates to be more sensitive to low temperatures whilst E198A isolates had no detectable fitness penalty.

*1.8.3.4. FRAC Group 3*

Another important class of fungicides used to manage *C. beticola* are DMIs. They have both protective and curative activity against *Cercospora* spp. and low levels of phytotoxicity (Brown et al., 1986; Dahmen and Staub, 1992). Although DMIs were initially thought to have a moderate risk of resistance development (Brown et al., 1986), *C. beticola* resistance has now been found in Europe (Karaoglanidis et al., 2001a), Morocco (El Housni et al., 2018), Canada (Trueman et al., 2017) and the U.S. (Bolton et al., 2012b; Rosenzweig et al., 2020; Secor et al., 2010). Resistance to DMIs is observed as a near-continuum, ranging from high to low $EC_{50}$ values (Karaoglanidis and Ioannidis, 2010). *C. beticola* isolates with $EC_{50}$ values of greater than 1 ppm caused significantly more disease on sugar beet after application of a DMI fungicide than isolates with $EC_{50}$ values below 1 ppm, implicating 1 ppm as a reasonable threshold value for DMI resistance (Bolton et al., 2012b). In the 2017 RRV region survey, 25.9% of tested *C. beticola* isolates were resistant ($EC_{50}$ > 1 ppm) to tetraconazole while 47.1% of the same isolates were resistant to another DMI difenoconazole, which suggests that there is not strict cross-resistance within the DMIs (Secor et al., 2017) and supports the earlier findings of (Karaoglanidis and Thanassoulopoulos, 2003). One study suggested that there may be some fitness penalties associated with DMI resistance, namely reduced virulence and spore production (Karaoglanidis et al., 2001b) but this has yet to be evidenced in field surveys.

The mechanism of resistance to DMIs is typically more complex than to benzimidazoles and QoIs. DMIs target the lanosterol 14α-demethylase CYP51 which is a cytochrome P450 enzyme catalyzing a key step in the fungal ergosterol biosynthesis pathway. Without the synthesis of the cell membrane sterol ergosterol, there is inhibition of fungal cell growth.

Resistance can occur not only through target site modifications of CYP51, but also through overexpression of CYP51, increased active efflux of DMIs and through multiple copies of the target CYP51 gene (Leroux et al., 2007; Ziogas and Malandrakis, 2015). In the RRV region of the U.S., *CbCYP51* was overexpressed in several *C. beticola* isolates with $EC_{50}$ values >1 ppm, in both qPCR and RNAseq studies, but the genetic mechanism underpinning this expression is unknown (Bolton et al., 2012a; Bolton et al., 2016). No evidence has been found for alternative splicing or differential methylation of *CbCYP51* between sensitive and resistance isolates (Bolton et al., 2012a). Interestingly, a silent mutation at codon 170 has been identified to be present only in highly resistant *C. beticola* isolates ($EC_{50}$ values >50 ppm) from Northern Greece (Nikou et al., 2009) and the RRV region of the U.S. ($EC_{50}$ values >20 ppm) (Obuya et al., 2015). Recently, non-synonymous polymorphisms in *CbCYP51* have also been discovered that appear to be linked to DMI resistance (Shrestha et al., 2020; Trkulja et al., 2017). The amino acid substitutions L144F, I309T, I387M and Y464S in isolates from the RRV region of the U.S. (Spanner and Bolton, *unpublished*) are all associated with DMI $EC_{50}$ values >1 ppm.

*1.8.3.5. FRAC Group 11*

The QoI class of fungicides were introduced in 1996 and first used for CLS in 2002, proving to be highly effective fungicides against *C. beticola* (Karadimos et al., 2005; Secor et al., 2010). Pathogen surveys in Europe (Birla et al., 2012; Piszczek et al., 2018), Morocco (El Housni et al., 2018), Japan (Kayamori et al., 2019), Canada (Trueman et al., 2013) and the U.S. (Kirk et al., 2012; Secor et al., 2010) have indicated the rapid development of resistance to QoIs, which appears to be stable. In 2017, 89.1% of *C. beticola* isolates surveyed in the RRV region of the U.S. were resistant to the QoI pyraclostrobin and therefore its use is no longer recommended for CLS management in the region (Secor et al., 2017).

QoIs act by binding the quinol oxidation site of the cytochrome bc1 complex in the mitochondria which disrupts ATP production (Fernández-Ortuño et al., 2008). The membrane protein cytochrome b forms the core of the complex and is encoded by the cytochrome b (*cytb*) gene. Similar to other fungi (Fernández-Ortuño et al., 2008), QoI-resistant isolates of *C. beticola* found to date have the substitution of glycine by alanine at codon 143 (designated G143A) (Birla et al., 2012; Bolton et al., 2013; Piszczek et al., 2018; Trkulja et al., 2017).

The identification of mutations underlying resistance to fungicide classes enables the rapid detection of resistance via PCR methods. Real-time PCR methods are already being employed for QoI resistance in annual surveys of *C. beticola* isolates (Bolton et al., 2013; Malandrakis et al., 2011) and methods have been developed to detect benzimidazole and DMI-resistant isolates (Nikou et al., 2009; Rosenzweig et al., 2015; Shrestha et al., 2020; Trkulja et al., 2013). A method of amplifying DNA under isothermal conditions has been developed and termed loop-mediated isothermal amplification or LAMP (Notomi et al., 2015). This tool may eventually allow for fungicide resistance profiling of a *C. beticola* field population prior to spraying to best determine the appropriate chemical regime.

### 1.9. Conclusions and future perspectives

Future studies of CLS disease in sugar beet can exploit the wealth of genetic and genomic resources that have become available in recent years for both *C. beticola* (de Jonge et al., 2018) and its hosts (del Río et al., 2019; Dohm et al., 2014). The development of gene editing technologies such as CRISPR-Cas9 (Doudna and Charpentier, 2014), which also revolutionized genetic editing in a broad variety of filamentous fungi as highlighted in multiple reviews (Idnurm and Meyer, 2018; Schuster and Kahmann, 2019; Song et al., 2019; Vicente et al., 2019), could be employed to investigate gene function via allele replacement in addition to gene knockouts. This would facilitate the identification of important genes and mutations for virulence in *C. beticola.*

Breeding for effective host resistance to CLS with minimal yield penalty will continue to be important and may be expedited by gene editing techniques if effective gene targets are identified. Additionally, since cercosporin is a virulence factor for the fungus, transferring a cassette of fungal-derived cercosporin AR genes to sugar beet could be a method to establish durable resistance in the host. Molecular advances will continue to help us understand additional aspects of CLS disease. The elucidation of the molecular basis of fungicide resistances is allowing us to both better monitor and manage resistant populations in the field so that we can maintain the efficacy of current available fungicides. It will be imperative in the future to identify key primary sources of inoculum and establish biological mechanisms used by *C. beticola* to generate genetic diversity. Ultimately, improved knowledge of host-pathogen interactions will aid in successful integrated management of CLS.

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# CHAPTER 2. GENOME-WIDE ASSOCIATION STUDIES REVEAL THE COMPLEX GENETIC ARCHITECTURE OF DMI FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA*[2]

## 2.1. Abstract

Cercospora leaf spot is the most important disease of sugar beet worldwide. The disease is caused by the fungus *Cercospora beticola* and is managed principally by timely application of fungicides including those of the sterol demethylation inhibitor (DMI) class. However, reliance on DMIs has caused an increase in resistance to this class of fungicides in multiple *C. beticola* populations. To better understand the genetic basis for resistance in *C. beticola*, a genome wide association study (GWAS) was conducted for the first time in this fungal pathogen. We performed whole genome resequencing of 190 *C. beticola* isolates predominantly from North Dakota and Minnesota that were phenotyped for sensitivity to tetraconazole, the most widely used DMI fungicide in this region. GWAS identified novel mutations on chromosomes one, four and nine associated with tetraconazole sensitivity: 5'UTR insertions in genes encoding a sorting nexin/RGS protein and a dual specificity DYRK protein kinase, mutations in an ABC multidrug resistance transporter and a SNP upstream of *CYP51*, the DMI target gene. This prompted haplotype analysis of *CYP51* which identified both synonymous (E170) and non-synonymous

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(L144F, I387M and Y464S) mutations associated with DMI resistance. Additionally, a putative codon bias effect for the L144F substitution was identified that generated different resistance potentials. Using radial plate growth as a fitness proxy, we did not find a trade-off associated with DMI fungicide resistance loci. We also identified a CYP51 paralog in *C. beticola*, CYP51-like, with high protein homology to CYP51C found uniquely in *Fusarium* species. *CYP51-like* does not appear to influence DMI sensitivity and exhibits partial gene absence in some isolates. Taken together, GWAS was an effective approach to identify novel mutations associated with DMI resistance that will prove critical for future fungicide resistance management programs.

## 2.2. Introduction

Demethylation inhibitor (DMI) compounds are effective antifungals in both medicine and agriculture for managing a broad range of fungal pathogens (Becher and Wirsel 2012). The DMIs, or azoles, inhibit fungi by interfering with sterol 14α-demethylase (Vanden Bossche et al. 1987), also known as cytochrome P450 monooxygenase family 51 (CYP51). Fungal CYP51 is required for synthesis of ergosterol, a key sterol component of fungal cell membranes required to maintain permeability and fluidity (Daum et al. 1998). DMIs have shown unique durability when compared to other single site fungicides, with control failures being rare even with widespread and prolonged use (Cools et al. 2013). However, resistance has still emerged in some fungal populations with long term exposure to DMIs, leading to reduced efficacy of the compounds in use (Staub 1991).

DMI resistance is often associated with changes to the molecular target, CYP51 (Becher and Wirsel 2012). Amino acid substitutions in CYP51 (Kelly et al. 1999a; Kelly et al. 1999b; Lamb et al. 2000; Snelders et al. 2011) or overexpression of *CYP51* (Carter et al. 2014; Ghosoph et al. 2007; Hamamoto et al. 2000; Ma et al. 2006; Villani et al. 2016) can lead to decreased DMI sensitivity. Some filamentous fungi have two or more paralogous *CYP51* genes

(Chen et al. 2020; Hawkins et al. 2014; Liu et al. 2011) which may result in an inherent reduction in DMI sensitivity, and allow these species to overcome some biological costs by restricting acquired resistance to one paralog (Becher and Wirsel 2012; Cools et al. 2013). Gain-of-function mutations in transcription factors (Dunkel et al. 2008; Liu et al. 2015) regulating ergosterol biosynthesis genes have also been linked to reduced DMI efficacy.

Non-CYP51 mechanisms of resistance can also be important in fungi. Such mechanisms include enhanced efflux of DMIs (Hahn and Leroch 2015) by plasma membrane-bound transporters in the multi-facilitator (MFS) or ATP-binding cassette (ABC) superfamilies (de Ramón-Carbonell et al. 2019; Hayashi et al. 2002b, a; Hellin et al. 2018; Leroux and Walker 2011; Zwiers et al. 2002), calcium signaling regulators (Edlind et al. 2002; Jain et al. 2003; Jia et al. 2009; Jia et al. 2012; Li et al. 2019; Zhang et al. 2013), the pleiotropic effect of melanization (Lendenmann et al. 2015) and other uncharacterized genes (Ballard et al. 2019).

DMI fungicides are currently integral for managing many important crop diseases (Price et al. 2015), including Cercospora leaf spot (CLS) disease of sugar beet (*Beta vulgaris* spp. *vulgaris*). CLS remains the most destructive foliar disease of sugar beet worldwide (Rangel et al. 2020). In addition to adequate host genetic tolerance and cultural practices to suppress the causal pathogen, timely fungicide applications are critical for disease management (Secor et al. 2010). The Red River Valley (RRV) region of North Dakota and Minnesota is the largest sugar beet production area in the US (NASS 2020) and has historically experienced huge economic losses due to CLS with large reductions in yield and the application of non-efficacious fungicides (Bolton et al. 2012a; Secor et al. 2010). The use of DMI fungicides in the RRV region began in 1999 when the Environmental Protection Agency granted an emergency exemption for sugar beet growers to use tetraconazole to manage CLS disease (Secor et al. 2010). Management of

fungicide resistance in the RRV has become a cooperative effort to maintain efficacy of fungicide groups (Khan and Smith 2005; Secor et al. 2010). This predominantly involves mixing and rotating fungicide chemistries within and between sprays, coupled with yearly assessment of fungicide sensitivities in *C. beticola*.

The magnitude of DMI resistance (measured by $EC_{50}$ values) in *C. beticola* and incidence of resistant isolates ($EC_{50}$ > 1µg/mL) in RRV field populations has steadily increased since 2006 (Rangel et al. 2020). Factors contributing to the rapid development of fungicide resistance in *C. beticola* are its polycyclic nature, high rate of sporulation and common spray programs used over large areas for disease management (Dekker 1986). Although experimental crosses of *C. beticola* are not possible to our knowledge, there is indirect population genetic evidence of sexual reproduction (Bolton et al. 2014; Bolton et al. 2012c; Groenewald et al. 2006b; Groenewald et al. 2008). In contrast, *C. beticola* populations from table beet exhibit microsatellites in linkage disequilibrium and do not always have equal mating type ratios, suggesting a mixed mode of reproduction (Knight et al. 2018; Knight et al. 2019; Vaghefi et al. 2016; Vaghefi et al. 2017). Evaluating levels of resistance is an important part of CLS fungicide resistance management (Secor et al. 2010) and has been aided by the development of PCR-based mutation detection tools to expedite the process (Birla et al. 2012; Bolton et al. 2012b; Shrestha et al. 2020). These tools have greatly increased the ability to effectively identify fungicide-resistant strains which enables growers to make educated fungicide application decisions based on the resistance profiles of their fields. However, molecular methods of resistance detection first require the elucidation of mutations involved in fungicide resistance. In *C. beticola,* overexpression of *CbCYP51* has been associated with high levels of DMI resistance in isolates from Greece (Nikou et al. 2009) and the US (Bolton et al. 2012b; Bolton et al. 2016). However, no upstream

insertions, duplications or transposable elements have been found that could be associated with gene overexpression (Bolton et al. 2012b; Nikou et al. 2009). Nikou et al. (2009) found that a single synonymous mutation at position E170 was associated with *CbCYP51* overexpression and DMI resistance. However, Obuya et al. (2015) could not demonstrate its involvement in DMI resistance through heterologous expression in yeast. Amino acid substitutions L144F, I387M and Y464S in CbCYP51 appeared to coincide with DMI resistance in *C. beticola* isolates from Serbia (Trkulja et al. 2017) but overall, it has been difficult to clearly associate any *CbCYP51* haplotype with resistance (Bolton et al. 2012b; Trkulja et al. 2017). Factors including small sample size may play a significant role in the interpretation of results in such studies.

A genome-wide association study (GWAS) is a powerful method for identifying genetic variants associated with complex traits such as DMI fungicide resistance (Sanglard 2019). This method can be performed using natural variation within a population and is a good alternative to biparental mapping when sexual crosses are not possible or convenient. The advent of cost-effective high-throughput sequencing technology is enabling whole genome sequencing for GWAS in a wide range of organisms (Power et al. 2017). Fungi are particularly amenable to GWAS due to their small genomes, haploidy and high rates of recombination (Falush 2016). GWAS has been successfully employed to identify loci associated with DMI resistance in several phytopathogenic fungi (Mohd-Assaad et al. 2016; Pereira et al. 2020; Talas et al. 2016). We hypothesized that GWAS would be an ideal strategy for finding genetic determinants underlying DMI resistance in *C. beticola*, a pathogen that cannot be experimentally crossed yet shows considerable genetic variation (Bolton et al. 2012c; Groenewald et al. 2006a; Groenewald et al. 2008; Moretti et al. 2006; Moretti et al. 2004; Rangel et al. 2020; Vaghefi et al. 2016). In this study, we aimed to unravel the genetic architecture of DMI fungicide resistance

in *C. beticola* by (i) performing whole-genome re-sequencing of 190 RRV *C. beticola* isolates and implementing an unbiased GWAS for resistance to the DMI fungicide tetraconazole, and (ii) establishing a Cas9-RNP gene editing method in *C. beticola* to functionally assess any potential fungicide resistance mutations.

## 2.3. Materials and methods

### 2.3.1. Field sampling of *C. beticola*

The 190 *C. beticola* isolates were collected from sugar beet leaves harvested from naturally infected commercial fields in the Red River Valley region of Minnesota and North Dakota, and Idaho (n=2), in 2016 (n=142) and 2017 (n=48) (Table S1). Conidia from the lesions on the sugar beet leaves were liberated into 30 µL sterile water (0.02% ampicillin) with a pipette tip and transferred to water agar plates (1.5% w/v agar (Sigma-Aldrich) and 0.02% ampicillin). Conidial germination occurred after 24 h at 22°C and then single conidia were transferred to Difco$^{TM}$ potato dextrose agar (PDA) plates which were incubated at 25°C for 14 days. These PDA plates served as source inoculum for all further studies for each of the 190 single spore-derived isolates. Of the 142 isolates collected in 2016, 62 were collected from two adjacent fields. Random representative sampling of strains was performed in these two fields, as outlined by McDonald (1997), by walking diagonally across the field and collecting a leaf every meter from a corner to the center of the field. Prior to fungicide application, 60 diseased leaves were harvested from each field and 100 leaves were harvested from each field post-application of tetraconazole (Eminent fungicide). All isolates collected from these two adjacent Fargo fields were genotyped using eight microsatellite markers to remove any potential clones, as described by Vaghefi et al. (2016), which led to the selection of 62 as part of the final population (n=190) (Table S1). The remaining isolates collected in 2016 (n=80) and 2017 (n=48) were obtained as

part of annual *C. beticola* fungicide resistance surveys in the RRV region, where growers send infected sugar beet leaves to the Secor lab at North Dakota State University for testing.

### 2.3.2. Phenotyping for DMI fungicide sensitivity

To measure sensitivity to the DMI fungicide active ingredient tetraconazole, $EC_{50}$ values were calculated from radial growth of the *C. beticola* isolates on amended media, as described by Secor and Rivera (2012). The single spore subcultures for all 190 isolates were transferred to clarified V8 (CV8) medium plates (10% v/v clarified V8 juice (Campbell's Soup Co.), 0.5% w/v $CaCO_3$, 1.5% w/v agar (Sigma-Aldrich)) and incubated at 20°C for 15 days in a continuous light regime. An agar plug of 4 mm in diameter was excised from the growing edge of the colony and placed in the center of a set of CV8 plates: one non-amended control plate and the rest amended with serial tenfold dilutions of technical grade tetraconazole (active ingredient of Eminent 125SL (Sipcam Agro)) from 0.001 to 100 µg/mL. All plates were incubated in the dark at 20 °C for 15 days after which two perpendicular measurements were made across the colonies and the diameter averaged. The percentage reduction in growth compared to the non-amended media was calculated for each tetraconazole concentration. The $EC_{50}$ value for each isolate was calculated by plotting the percentage reduction in growth against logarithmic tetraconazole concentration and using regression curve fitting to find the tetraconazole concentration that reduced growth by 50%. Statistical analysis was comprised of one-way ANOVA followed by a post-hoc Tukey test to identify significant differences between groups.

### 2.3.3. Radial growth assays

All 190 isolates were grown on CV8 plates for 15 days at 20°C in a continuous light regime, as described above. An agar plug of 4mm in diameter was taken from the leading edge of these cultures and transferred to a new CV8 plate. Three CV8 plates were initiated per isolate, and these were grown at 23°C under continuous light for 16 days. The radius of each culture was

measured after 2, 6, 9, 13 and 16 days and a mean value was calculated for each day. Linear regression of radius (mm) versus time (days) was used to establish the rate of radial growth in mm/day. Three CV8 plates amended with 1M NaCl were also initiated per isolate and grown under the same conditions. The radius was measured for these cultures after 6, 9, 12, 16, 20 and 23 days and a mean value was calculated for each day. Linear regression of radius (mm) versus time (days) was used to establish the rate of radial growth in mm/day for both unamended and salt stress conditions.

### 2.3.4. DNA extraction and whole genome resequencing

High quality genomic DNA was extracted for library preparation from liquid cultures of *C. beticola*, A single 6 mm agar plug excised from the source PDA plate was sliced into small pieces and used to inoculate 100 mL Difco™ potato dextrose broth (PDB). Cultures were grown at 25 ºC for seven days, shaking at 150 rpm. The mycelia was filtered through miracloth, flash frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. The method of Zhang et al. (1996) was followed for large scale isolation of genomic DNA but replacing the chloroform:isoamyl (24:1) with phenol:chloroform:isopropanol (25:24:1). The resultant DNA was then cleaned up further using the Qiagen DNeasy Plant Mini Kit (Cat No. 69106) according to manufacturer's instructions. DNA samples were sent to Beijing Genome Institute (BGI) for library preparation (400 bp inserts) and 100 bp or 150 bp paired-end whole genome resequencing using the Illumina HiSeq 4000 platform to achieve approximately 25X genome coverage per isolate.

### 2.3.5. Variant calling

Sequencing read quality was analyzed using FastQC (Andrews 2017) and Trimmomatic (Bolger et al. 2014) was subsequently used to trim reads (HEADCROP:10) and remove unpaired reads. The trimmed reads were aligned to the reference *C. beticola* 09-40 genome (NCBI RefSeq

assembly GCF_002742065.1) (de Jonge et al. 2018) using BWA-MEM (Li 2013). SAMtools (Li et al. 2009) was used to convert the output sam files to sorted, indexed bam files and to index the reference genome. Duplicate reads (PCR and optical) were removed from bam files using Picard MarkDuplicates (Institute 2016). Genome Analysis Tool Kit (GATK) V4.0.8.1 HaplotypeCaller (McKenna et al. 2010) was used to identify SNPs and small indels between each isolate and the 09-40 Reference sequence. GATK CombineGVCFs was used to combine all HaplotypeCaller gVCFs into a multi-sample gVCF which was genotyped by GATK GenotypeGVCFs to produce the final VCF. Vcftools (Danecek et al. 2011) was then used to filter variants for genotyping quality (--minGQ 10) and sequencing depth (--minDP 3).

**2.3.6. Population structure and LD decay analyses**

Before performing principle component analysis (PCA), the VCF was filtered using Vcftools to retain SNPs only (--remove-indels). Plink (Chang et al. 2015) was used to prune the SNPs for linkage disequilibrium (LD), with option –indep-pairwise 50 10 0.1, to analyze pairwise association between SNPs ($r^2$) in chromosomal windows of 50 SNPs at a time and removing pairs with $r^2 > 0.1$ before shifting the window 10bp. PCA was performed using the SMARTPCA function as part of the EIGENSOFT package (Patterson et al. 2013) and plots of PCs were created using PCAviz (Novembre et al. 2018).

To assess LD decay within the mapping population, the PopLDdecay software version 3.40 (Zhang et al. 2019) was used to estimate pairwise linkage disequilibrium ($R^2$) between all SNPs within 10 kb of each other and this was plotted as LD decay.

**2.3.7. Association mapping**

Association mapping was performed using GAPIT v3.0 (Wang and Zhang 2018). The imported genotyping vcf was first filtered in TASSEL v5.0 (Bradbury et al. 2007) to convert heterozygous calls to missing data and to establish a minor allele frequency of 0.05 and

minimum SNP count of 171/190 (10% missing). As the tetraconazole $EC_{50}$ phenotype was highly positive skewed (not normally distributed), all values were $log_{10}$ transformed prior to association mapping. A general linear model was run as a naïve model and also as a model incorporating the optimal number of components derived from PCA as fixed effects to correct for population structure. GAPIT BIC was used to determine the optimum number of PCs for the model. A mixed linear model was selected for both tetraconazole sensitivity and radial growth phenotypes, incorporating a kinship matrix (K, calculated using the default VanRaden algorithm) as a random effect. The most robust model for a trait association was selected through visualization of the quantile-quantile (Q-Q) plots, which show the relationship between observed and expected p-values. The R package qqman (Turner 2014) was used to generate Manhattan and Q-Q plots. Allelic effect estimates for phenotypes were derived from association mapping in GAPIT. R v.4.0.2 was used for the Pearson's product-moment correlation test.

### 2.3.8. *CYP51* gene sequencing

*C. beticola* isolates were sampled and single-spored from the Red River Valley region of North Dakota and Minnesota in 2019 (n=52), as described above. DNA was extracted directly from PDA cultures using a quick sodium dodecyl sulfate (SDS) lysis prep (Fran Lopez Ruiz, personal communication). The entire *CYP51* gene sequence (NCBI XP_023450255.1) was amplified in PCR with primers 530 and 532 from Bolton et al. (2012b), using standard conditions. Sanger sequencing of the entire PCR product was carried out using external primers 530 and 532, and internal primers 426, 349 and 566 from Bolton et al. (2012b).

### 2.3.9. Transcription factor binding site prediction

The JASPAR CORE fungal database (www.jaspar.genereg.net/) (Fornes et al. 2020) containing experimentally-determined preferred DNA binding sites of transcription factors was queried with two different 21 bp sequences upstream of *CYP51*, which had the C or T SNP at

9_1452111 in the middle of the sequence: AATGTTGAAACCGTACGATGG (sensitive) and AATGTTGAAATCGTACGATGG (resistant). The 184 non-redundant matrix models from fungi were used to scan the two sequences using a relative profile score threshold of 80%, and differential results were reported.

### 2.3.10. Codon usage assessment

Predicted coding sequences (CDS) for the 09-40 *C. beticola* reference were downloaded from NCBI (RefSeq assembly accession GCF_002742065.1) and entered into the Codon Usage tool in the Sequence Manipulation Suite (Stothard 2000) in order to calculate number and frequency of each codon type.

### 2.3.11. Phylogenetic analysis

Ascomycete CYP51 protein sequences (including all paralogs CYP51A, B and C when present in a species) were obtained from DOE Joint Genome Institute (JGI) or NCBI protein databases along with *Saccharomyces cerevisiae* ERG11 and *Homo sapiens* CYP7A1, which was used as an outgroup. Amino acid sequence alignment was performed in MUSCLE 3.8.31 (Edgar 2004) and the alignment was refined using Gblocks 0.91b (Castresana 2002). Maximum-likelihood phylogenetic analysis was executed in PhyML 3.0 (Guindon et al. 2010) and TreeDyn 198.3 was used to render the final tree (Chevenet et al. 2006). All tools were implemented using the phylogeny.fr platform (Dereeper et al. 2008).

### 2.3.12. Cas9 RNP-mediated gene editing

The method used was adapted from Foster et al. (2018). SgRNAs were selected using the E-CRISP program (http://www.e-crisp.org/E-CRISP/) (Table S5) and then BLASTn was performed against the *C. beticola* 09-40 reference genome to ensure the absence of off-target binding sites. Oligonucleotides for sgRNA synthesis were designed using NEB's online tool (http://nebiocalculator.neb.com/#!/sgrna). SgRNAs were synthesized *in vitro* using the EnGen

sgRNA synthesis kit, *S. pyogenes* (NEB #E3322) and were purified and concentrated into 25 µL using the RNA Clean & Concentrator-25 kit (Zymo Research #R1017). SgRNAs were complexed to 6 µg EnGen Spy Cas9-NLS (NEB #M0646) in an approximately 1:1 molar ratio for 15 min at RT. Donor DNA for introducing targeted SNPs was an 81 bp oligonucleotide with the desired single bp change in the center and 40 bp flanks of homologous sequence (Table S5). Forward and reverse complement versions of the donor oligonucleotide were complexed together at 95°C for five min prior to transformation. For co-editing, the *Hph* hygromycin resistance gene was amplified from pDAN (Friesen et al. 2006) using oligonucleotides with an additional 5' 40 bp of homologous sequence flanking the desired locus for insertion (Table S5) and was purified and concentrated using ethanol-sodium acetate precipitation. Protoplasts were generated from cultures of benzimidazole-sensitive strain 16-1124 and DMI-sensitive strain 16-100 as described in Bolton et al. (2016). The complexed Cas9-RNP was added to 150 µL of protoplasts at $10^8$/mL concentration alongside 2 µL donor DNA (for co-editing 2 x Cas9-RNP were added at half concentration, one to create the SNP change in *CYP51* and the other to insert *Hph* simultaneously at another locus) and the protoplasts were incubated on ice for 30 min. The remainder of the procedure starting from PEG addition occurred as described by Liu and Friesen (2012). The only changes were in the addition of compounds for selective growth in the regeneration media agar: 10 µg/mL topsin (for E198A in beta-tubulin editing attempts) and 5-10 µg/mL tetraconazole (for *CYP51* editing attempts). Putative transformants growing on the surface of the agar after ~5-10 days at RT were isolated and DNA extracted to perform Sanger sequencing over the relevant intended mutation.

## 2.4. Results

### 2.4.1. Field sampling of *C. beticola* isolates and selection for genome sequencing

We initially set out to investigate the genetic architecture of DMI fungicide resistance in *C. beticola* isolates collected from two adjacent fields near Fargo, North Dakota in 2016. Since previous studies have demonstrated that *C. beticola* populations have substantial genetic variation (Groenewald et al. 2007; Moretti et al. 2006; Moretti et al. 2004), even exhibiting multiple genotypes on a single leaf (Bolton et al. 2012c), we reasoned that isolates collected from two fields would harbor enough genetic variation for GWAS. Approximately 300 isolates were clone-corrected using eight microsatellite markers and unique isolates were subsequently phenotyped for tetraconazole sensitivity by calculating $EC_{50}$ values. From these isolates, a population of 62 unique *C. beticola* isolates was selected based on tetraconazole $EC_{50}$ value: approximately half (34 isolates) had a tetraconazole $EC_{50}$ value of below 1.0 µg/mL (arbitrarily considered sensitive) (Bolton et al. 2012a) and the other half (28 isolates) had an $EC_{50}$ value of 1.0 µg/mL or higher and were considered resistant (Table 2.1). These 62 isolates underwent Illumina whole genome resequencing with an aim of 25X or greater genome coverage. After filtering for genotype quality and read depth, 672,149 genetic variants (SNPs and indels) were identified. All GWAS attempts failed to produce models that corrected for population structure adequately and thus generated either highly overinflated or underinflated *p*-values (not shown). To overcome this, we sequenced an additional 128 isolates that were collected from different infected commercial fields throughout the Red River Valley (RRV) region of Minnesota and North Dakota, except two isolates collected from Idaho, during field surveys in 2016 (n=80) and 2017 (n=48) (Table 2.1). These isolates were selected due to their more diverse geographic origins within the RRV and more extreme tetraconazole sensitivities. In total, 190 isolates of *C. beticola* were used in this study (Table 2.1).

$EC_{50}$ values were calculated for all 190 isolates to tetraconazole, the active ingredient of Eminent fungicide which is widely used in the RRV region. The highest $EC_{50}$ value measured was the maximum concentration used of 100 µg/mL indicating that the true value could be greater still (Table 2.1). The lowest $EC_{50}$ obtained was 0.008 µg/mL, comparable to $EC_{50}$ values of *C. beticola* isolates collected in the RRV region in 1997-98 (Bolton et al. 2012a).

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study. All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 1 | 16-F1 | Minn-Dak_Fargo | 0.569 | 2.182 | 0.595 |
| 2 | 16-F2 | Minn-Dak_Fargo | 0.542 | 1.813 | 0.497 |
| 3 | 16-F3 | Minn-Dak_Fargo | 0.083 | 1.791 | 1.001 |
| 4 | 16-F4 | Minn-Dak_Fargo | 0.521 | 1.807 | 0.840 |
| 5 | 16-F5 | Minn-Dak_Fargo | 0.116 | 2.059 | 0.622 |
| 6 | 16-F6 | Minn-Dak_Fargo | 0.585 | 1.680 | 0.571 |
| 7 | 16-F7 | Minn-Dak_Fargo | 0.567 | 2.138 | 0.375 |
| 9 | 16-F9 | Minn-Dak_Fargo | 0.553 | 2.022 | 0.540 |
| 11 | 16-F11 | Minn-Dak_Fargo | 0.546 | 1.727 | 0.573 |
| 12 | 16-F12 | Minn-Dak_Fargo | 0.503 | 1.853 | 0.558 |
| 13 | 16-F13 | Minn-Dak_Fargo | 0.354 | 2.029 | 0.824 |
| 14 | 16-F14 | Minn-Dak_Fargo | 0.579 | 1.631 | 0.593 |
| 15 | 16-F15 | Minn-Dak_Fargo | 0.528 | 1.310 | 0.555 |
| 16 | 16-F16 | Minn-Dak_Fargo | 0.063 | 1.582 | 0.804 |
| 17 | 16-F17 | Minn-Dak_Fargo | 0.637 | 1.824 | 0.726 |
| 18 | 16-F18 | Minn-Dak_Fargo | 0.560 | 1.764 | 0.564 |
| 19 | 16-F19 | Minn-Dak_Fargo | 0.428 | 1.339 | 0.524 |
| 20 | 16-F20 | Minn-Dak_Fargo | 0.509 | 0.879 | 0.467 |
| 21 | 16-F21 | Minn-Dak_Fargo | 0.517 | 1.600 | 0.415 |
| 22 | 16-F22 | Minn-Dak_Fargo | 0.740 | 2.166 | 1.061 |
| 23 | 16-F23 | Minn-Dak_Fargo | 38.107 | 1.889 | 0.377 |
| 24 | 16-F24 | Minn-Dak_Fargo | 1.929 | 1.761 | 0.584 |
| 25 | 16-F25 | Minn-Dak_Fargo | 1.741 | 1.676 | 0.578 |
| 26 | 16-F26 | Minn-Dak_Fargo | 0.503 | 1.950 | 0.473 |
| 27 | 16-F27 | Minn-Dak_Fargo | 5.441 | 1.838 | 0.626 |
| 28 | 16-F28 | Minn-Dak_Fargo | 2.275 | 1.961 | 0.539 |
| 29 | 16-F29 | Minn-Dak_Fargo | 0.561 | 1.302 | 0.500 |
| 30 | 16-F30 | Minn-Dak_Fargo | 0.585 | 1.651 | 0.592 |
| 31 | 16-F31 | Minn-Dak_Fargo | 2.914 | 1.404 | 0.686 |
| 32 | 16-F32 | Minn-Dak_Fargo | 0.443 | 1.687 | 0.677 |
| 33 | 16-F33 | Minn-Dak_Fargo | 6.021 | 1.265 | 0.747 |
| 34 | 16-F34 | Minn-Dak_Fargo | 8.158 | 1.909 | 0.529 |
| 35 | 16-F35 | Minn-Dak_Fargo | 36.549 | 1.652 | 0.850 |
| 36 | 16-F36 | Minn-Dak_Fargo | 6.070 | 2.114 | 0.881 |
| 37 | 16-F37 | Minn-Dak_Fargo | 31.584 | 2.129 | 0.670 |
| 38 | 16-F38 | Minn-Dak_Fargo | 43.903 | 1.863 | 0.636 |

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study (continued). All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 39 | 16-F39 | Minn-Dak_Fargo | 8.921 | 2.106 | 0.735 |
| 40 | 16-F40 | Minn-Dak_Fargo | 0.598 | 2.036 | 0.464 |
| 41 | 16-F41 | Minn-Dak_Fargo | 9.596 | 1.274 | 0.708 |
| 42 | 16-F42 | Minn-Dak_Fargo | 5.276 | 2.038 | 0.887 |
| 43 | 16-F43 | Minn-Dak_Fargo | 10.537 | 1.502 | 0.639 |
| 44 | 16-F44 | Minn-Dak_Fargo | 0.988 | 1.55 | 0.275 |
| 45 | 16-F45 | Minn-Dak_Fargo | 9.708 | 1.501 | 0.706 |
| 46 | 16-F46 | Minn-Dak_Fargo | 0.932 | 1.404 | 0.596 |
| 47 | 16-F47 | Minn-Dak_Fargo | 5.12 | 2.524 | 0.819 |
| 48 | 16-F48 | Minn-Dak_Fargo | 61.118 | 1.168 | 0.735 |
| 49 | 16-F49 | Minn-Dak_Fargo | 5.8 | 2.286 | 0.656 |
| 50 | 16-F50 | Minn-Dak_Fargo | 0.964 | 1.889 | 0.578 |
| 51 | 16-F51 | Minn-Dak_Fargo | 0.955 | 1.456 | 0.786 |
| 52 | 16-F52 | Minn-Dak_Fargo | 7.661 | 2.158 | 0.752 |
| 53 | 16-F53 | Minn-Dak_Fargo | 10 | 1.15 | 0.526 |
| 54 | 16-F54 | Minn-Dak_Fargo | 41.295 | 2.284 | 0.841 |
| 55 | 16-F55 | Minn-Dak_Fargo | 0.71 | 1.046 | 0.431 |
| 56 | 16-F56 | Minn-Dak_Fargo | 5.367 | 2.354 | 0.649 |
| 57 | 16-F57 | Minn-Dak_Fargo | 80.749 | 0.739 | 0.514 |
| 58 | 16-F58 | Minn-Dak_Fargo | 61.722 | 1.308 | 0.748 |
| 59 | 16-F59 | Minn-Dak_Fargo | 0.65 | 1.668 | 0.48 |
| 61 | 16-16 | SMBSC | 31.945 | 1.985 | 0.719 |
| 62 | 16-45 | SMBSC | 19.361 | 2.264 | 0.604 |
| 63 | 16-46 | SMBSC | 59.412 | 1.785 | 0.555 |
| 64 | 16-62 | Unknown | 12.331 | 1.988 | 0.5 |
| 65 | 16-92 | SMBSC | 64.56 | 1.737 | 0.819 |
| 66 | 16-131 | ACS_EGF | 6.653 | 1.532 | 0.407 |
| 67 | 16-175 | SMBSC | 83.875 | 2.181 | 1.453 |
| 68 | 16-339 | ACS_Drayton | 0.067 | 1.451 | 0.273 |
| 69 | 16-591 | ACS_EGF | 0.395 | 1.945 | 0.461 |
| 71 | 16-665 | Minn-Dak_MN | 0.371 | 1.781 | 1.224 |
| 72 | 16-735 | Minn-Dak_MN | 0.403 | 1.684 | 0.579 |
| 73 | 16-781 | Minn-Dak_MN | 0.205 | 1.609 | 0.825 |
| 74 | 16-787 | Minn-Dak_MN | 0.455 | 1.384 | 0.524 |

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study (continued). All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 75 | 16-805 | ACS_Moorhead | 0.404 | 1.166 | 0.722 |
| 76 | 16-810 | ACS_Moorhead | 0.4 | 2.242 | 0.784 |
| 77 | 16-890 | SMBSC | 55.224 | 2.336 | 1.22 |
| 78 | 16-1148 | ACS_Crookston | 6.362 | 1.653 | 0.813 |
| 79 | 16-26 | SMBSC | 34.512 | 1.224 | 0.508 |
| 80 | 16-33 | SMBSC | 0.094 | 1.629 | 0.551 |
| 81 | 16-72 | SMBSC | 29.821 | 1.859 | 0.522 |
| 82 | 16-93 | SMBSC | 65.37 | 1.711 | 1.176 |
| 83 | 16-317 | ACS_Drayton | 77.542 | 1.724 | 0.888 |
| 84 | 16-414 | SMBSC | 65.161 | 1.13 | 0.634 |
| 85 | 16-498 | ACS_Hillsboro | 6.622 | 1.633 | 0.783 |
| 86 | 16-523 | ACS_Hillsboro | 5.889 | 2.003 | 0.773 |
| 87 | 16-665 | Minn-Dak_MN | 0.371 | 1.875 | 0.623 |
| 88 | 16-704 | Minn-Dak_MN | 0.415 | 1.681 | 0.378 |
| 89 | 16-716 | Minn-Dak_MN | 0.451 | 1.912 | 0.527 |
| 90 | 16-722 | Minn-Dak_MN | 0.436 | 1.56 | 0.799 |
| 91 | 16-724 | Minn-Dak_MN | 0.412 | 1.342 | 0.636 |
| 92 | 16-790 | ACS_Moorhead | 83.546 | 2.211 | 0.818 |
| 93 | 16-819 | ACS_Moorhead | 63.83 | 1.541 | 0.719 |
| 94 | 16-868 | ACS_EGF | 6.579 | 1.507 | 0.87 |
| 95 | 16-1105 | ACS_EGF | 5.841 | 1.877 | 1.315 |
| 96 | 16-1169 | ACS_Drayton | 6.302 | 1.147 | 0.594 |
| 97 | 16-F61 | Minn-Dak_Fargo | 0.46 | 1.776 | 0.78 |
| 98 | 16-F62 | Minn-Dak_Fargo | 9.03 | 1.404 | 0.826 |
| 99 | 16-F63 | Minn-Dak_Fargo | 0.55 | 2.056 | 0.334 |
| 100 | 16-F64 | Minn-Dak_Fargo | 0.52 | 1.275 | 0.406 |
| 101 | 16-F65 | Minn-Dak_Fargo | 6.57 | 1.829 | 0.606 |
| 102 | 16-100 | SMBSC | 0.08 | 1.068 | 0.457 |
| 103 | 16-1022 | ACS_Crookston | 27.639 | 1.342 | 1.127 |
| 104 | 16-1083 | SMBSC | 7.258 | 2.241 | 0.898 |
| 105 | 16-1110 | ACS_EGF | 0.072 | 1.894 | 0.827 |
| 106 | 16-1124 | SMBSC | 0.081 | 1.949 | 0.622 |
| 107 | 16-1129 | SMBSC | 18.33 | 1.38 | 0.504 |

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study (continued). All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 108 | 16-1138 | ACS_Crookston | 0.07 | 1.561 | 0.505 |
| 109 | 16-1151 | ACS_Crookston | 11 | 1.599 | 0.805 |
| 110 | 16-1155 | ACS_Crookston | 0.073 | 1.821 | 0.603 |
| 111 | 16-1211 | ACS_Drayton | 0.076 | 1.629 | 0.499 |
| 112 | 16-1214 | ACS_Drayton | 0.076 | 1.18 | 0.434 |
| 114 | 16-123 | BASF | 0.095 | 1.378 | 0.675 |
| 115 | 16-141 | ACS_Moorhead | 0.09 | 1.687 | 0.483 |
| 116 | 16-163 | ACS_Moorhead | 0.083 | 2.381 | 1.098 |
| 117 | 16-1044 | ACS_EGF | 0.512 | 1.592 | 0.46 |
| 118 | 16-181 | SMBSC | 40.302 | 2.073 | 0.761 |
| 119 | 16-186 | SMBSC | 0.091 | 1.713 | 0.842 |
| 120 | 16-224 | Minn-Dak_MN | 65.277 | 1.711 | 0.853 |
| 121 | 16-270 | Minn-Dak_MN | 65.26 | 1.927 | 0.741 |
| 122 | 16-29 | SMBSC | 87.739 | 2.169 | 0.946 |
| 123 | 16-326 | ACS_Drayton | 0.098 | 1.567 | 0.945 |
| 124 | 16-351 | ACS_Drayton | 57.213 | 1.842 | 0.63 |
| 125 | 16-36 | SMBSC | 0.081 | 1.235 | 0.606 |
| 126 | 16-363 | ACS_Drayton | 0.082 | 1.592 | 0.355 |
| 127 | 16-374 | ACS_Drayton | 55.56 | 1.971 | 0.655 |
| 128 | 16-412 | SMBSC | 48.483 | 1.319 | 0.667 |
| 129 | 16-438 | ACS_Crookston | 0.088 | 1.377 | 0.523 |
| 130 | 16-451 | ACS_Crookston | 65.927 | 2.039 | 0.938 |
| 131 | 16-503 | ACS_Hillsboro | 0.092 | 1.88 | 0.728 |
| 132 | 16-54 | SMBSC | 74.127 | 1.929 | 0.781 |
| 133 | 16-546 | ACS_Hillsboro | 18.033 | 1.354 | 0.883 |
| 134 | 16-569 | ACS_EGF | 81.667 | 2.112 | 0.816 |
| 135 | 16-638 | ACS_EGF | 0.075 | 1.463 | 0.637 |
| 136 | 16-723 | Minn-Dak_MN | 73.782 | 2.048 | 0.863 |
| 137 | 16-731 | Minn-Dak_MN | 48.907 | 1.976 | 0.903 |
| 138 | 16-795 | ACS_Moorhead | 11.942 | 2.088 | 0.574 |
| 139 | 16-827 | ACS_Moorhead | 0.081 | 2.111 | 0.245 |
| 140 | 16-842 | ACS_Crookston | 79.556 | 1.437 | 0.818 |
| 141 | 16-879 | SMBSC | 70.707 | 2.129 | 0.459 |

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study (continued). All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 142 | 16-90 | SMBSC | 78.959 | 0.967 | 0.744 |
| 143 | 16-938 | ACS_Drayton | 72.796 | 1.757 | 0.694 |
| 144 | 16-946 | ACS_Drayton | 57.83 | 1.677 | 1.13 |
| 145 | 16-987 | ACS_Drayton | 0.098 | 1.649 | 0.311 |
| 146 | 17-1015 | SMBSC | 75.57 | 1.422 | 0.309 |
| 147 | 17-1045 | ACS_EGF | 98.66 | 1.842 | 0.635 |
| 148 | 17-1047 | ACS_EGF | 0.034 | 1.976 | 0.332 |
| 149 | 17-1070 | ACS_Crookston | 0.061 | 1.804 | 0.509 |
| 150 | 17-1096 | ACS_Drayton | 0.056 | 1.513 | 0.547 |
| 151 | 17-1109 | ACS_Hillsboro | 0.058 | 2.076 | 0.432 |
| 152 | 17-1121 | ACS_Crookston | 68.88 | 1.905 | 1.021 |
| 153 | 17-1127 | ACS_Drayton | 0.062 | 1.576 | 0.634 |
| 154 | 17-1132 | ACS_Drayton | 58.81 | 2.307 | 0.483 |
| 155 | 17-1162 | SMBSC | 76.2 | 1.89 | 0.32 |
| 156 | 17-132 | Minn-Dak_MN | 0.072 | 1.614 | 0.55 |
| 157 | 17-177 | ACS_Drayton | 0.091 | 1.819 | 0.553 |
| 158 | 17-214 | ACS_Drayton | 0.008 | 2.125 | 0.481 |
| 159 | 17-235 | ACS_Drayton | 0.008 | 1.8 | 0.529 |
| 160 | 17-251 | ACS_Drayton | 0.062 | 1.493 | 0.556 |
| 161 | 17-300 | Minn-Dak_MN | 38.781 | 2.309 | 0.705 |
| 162 | 17-307 | Minn-Dak_MN | 0.077 | 2.282 | 0.614 |
| 163 | 17-354 | SMBSC | 65.364 | 1.733 | 0.541 |
| 164 | 17-363 | SMBSC | 0.063 | 1.605 | 0.572 |
| 165 | 17-385 | ACS_Moorhead | 0.093 | 1.179 | 0.422 |
| 166 | 17-390 | SMBSC | 72.84 | 1.26 | 0.787 |
| 167 | 17-410 | SMBSC | 0.083 | 1.467 | 0.503 |
| 168 | 17-443 | SMBSC | 0.088 | 1.543 | 0.557 |
| 169 | 17-478 | ACS_EGF | 0.06 | 1.674 | 0.459 |
| 170 | 17-48 | ID | 81.087 | 1.411 | 0.732 |
| 171 | 17-51 | ID | 0.082 | 1.522 | 1.106 |
| 172 | 17-516 | Minn-Dak_MN | 73.124 | 1.774 | 0.494 |
| 173 | 17-528 | Minn-Dak_MN | 0.077 | 1.909 | 0.471 |
| 174 | 17-585 | Minn-Dak_MN | 0.08 | 2.346 | 0.705 |

Table 2.1. The numbers, names, sampling location and phenotyping data for all 190 *C. beticola* isolates used in this study (continued). All isolates were phenotyped for sensitivity to tetraconazole ($EC_{50}$ measurements) and rate of growth (mm/day) on unamended potato dextrose agar media.

| Isolate number[1] | Isolate name[2] | Sampling location[3] | Tetraconazole EC50[4] | Radial growth rate (mm/day)[5] | Radial growth rate under salt stress (mm/day)[6] |
|---|---|---|---|---|---|
| 175 | 17-594 | Minn-Dak_MN | 50.235 | 2.046 | 0.807 |
| 176 | 17-633 | ACS_Hillsboro | 59.885 | 2.039 | 0.772 |
| 177 | 17-661 | ACS_Hillsboro | 77.5 | 1.852 | 0.685 |
| 178 | 17-696 | ACS_Crookston | 0.008 | 1.75 | 0.655 |
| 179 | 17-724 | ACS_EGF | 35.714 | 2.205 | 0.548 |
| 180 | 17-741 | ACS_EGF | 0.008 | 1.692 | 0.57 |
| 181 | 17-760 | ACS_Hillsboro | 54.563 | 1.577 | 0.526 |
| 182 | 17-799 | ACS_Drayton | 0.07 | 1.809 | 0.465 |
| 183 | 17-82 | SMBSC | 58.507 | 1.813 | 0.458 |
| 184 | 17-837 | ACS_Hillsboro | 100 | 1.813 | 0.904 |
| 185 | 17-852 | ACS_Hillsboro | 70.898 | 1.997 | 0.805 |
| 186 | 17-869 | ACS_Hillsboro | 0.096 | 2.154 | 0.59 |
| 187 | 17-912 | ACS_EGF | 46.362 | 2.187 | 0.975 |
| 188 | 17-933 | ACS_EGF | 0.51 | 1.703 | 0.547 |
| 189 | 16-1170 | ACS_Drayton | 0.076 | 1.971 | 0.241 |
| 190 | 16-892 | SMBSC | 0.084 | 1.736 | 0.462 |
| 191 | 17-1087 | ACS_Drayton | 52.75 | 1.972 | 0.435 |
| 192 | 17-256 | ACS_Drayton | 0.07 | 1.555 | 0.946 |
| 193 | 17-432 | SMBSC | 19.501 | 1.34 | 0.544 |
| 194 | 17-501 | Minn-Dak_MN | 58.843 | 1.43 | 0.662 |
| 195 | 17-950 | ACS_EGF | 83.29 | 2.123 | 0.935 |

[1]Numbers used to refer to individual isolates in PCA plots and other analysis performed in this study.
[2]Isolate names that will be used for upload to NCBI short read archive. 16/17 refers to the year of isolate collection (2016/17).
[3]Indicates factory district from which isolate was collected. ACS = American Crystal Sugar. SMBSC = Southern Minnesota Beet Sugar Cooperative. Minn-Dak = Minn-Dak Farmers Cooperative. BASF = BASF chemicals company.
[4]Effective concentration to reduce growth by 50% ($EC_{50}$) values were calculated for tetraconazole as described by Secor and Rivera (2012).
[5]Radial growth rate for each isolate in mm/day was calculated by growing them on CV8 plates and using linear regression for mean measurements taken at 2, 6, 9, 13 and 16 days.
[6]Radial growth for each isolate under salt stress in mm/day was calculated by growing them on CV8 plates amended with 1M NaCl and using linear regression for mean measurements taken at 6, 9, 13, 16, 20 and 23 days.

### 2.4.2. Genome sequencing of *C. beticola* isolates

To map the genetic architecture of resistance to DMI fungicides, we performed whole genome resequencing of 190 *C. beticola* isolates. We mapped Illumina reads of each isolate to the 09-40 reference genome (de Jonge et al. 2018) (NCBI RefSeq assembly GCF_002742065.1). The resulting coverage per genome ranged from 18X to 40X with a mean coverage of 32X (Table 2.2). GATK HaplotypeCaller was used to call SNPs and indels against the reference genome for each isolate. We used the default diploid ploidy level, instead of -ploidy 1 option in our haploid fungus, to allow us to filter out variants in any poorly aligned regions that result in heterozygous calls. After filtering for genotype quality and read depth, 868,218 variants were identified including 732,852 SNPs, corresponding to an average SNP density of ~20 SNPs per kb. All heterozygous sites were transformed to missing data. For genome-wide association mapping, the identified SNPs/indels were filtered further. A minor allele frequency of 0.05 reduced variants to 424,456, eliminating over half of called variants. Filtering for 10% missing data reduced the total number to 320,530 variants.

Table 2.2. Genome sequencing statistics for all 190 *C. beticola* isolates in this study.

| Isolate Name | Total number of reads | Sequence length (bp) | Reference genome alignment rate (%) | Mean reference genome coverage (X) |
|---|---|---|---|---|
| 16-F1 | 12718465 | 100 | 95.87 | 33 |
| 16-F2 | 13166029 | 100 | 95.47 | 34 |
| 16-F3 | 10804832 | 100 | 94.42 | 28 |
| 16-F4 | 12773284 | 100 | 96.6 | 33 |
| 16-F5 | 9502166 | 100 | 96.18 | 25 |
| 16-F6 | 11783600 | 100 | 96.62 | 31 |
| 16-F7 | 11936524 | 100 | 96.35 | 31 |
| 16-F9 | 12795308 | 100 | 96.01 | 33 |
| 16-F11 | 11072823 | 100 | 95.66 | 29 |
| 16-F12 | 11310699 | 100 | 95.88 | 29 |
| 16-F13 | 8903227 | 100 | 96.8 | 23 |
| 16-F14 | 11152696 | 100 | 96.66 | 29 |
| 16-F15 | 9859917 | 100 | 96.66 | 26 |
| 16-F16 | 14266731 | 100 | 96.76 | 37 |
| 16-F17 | 10947158 | 100 | 96.2 | 28 |
| 16-F18 | 10098019 | 100 | 96.41 | 26 |
| 16-F19 | 12044026 | 100 | 91.03 | 30 |
| 16-F20 | 11250660 | 100 | 96.44 | 29 |
| 16-F21 | 11589045 | 100 | 96.08 | 30 |
| 16-F22 | 12556783 | 100 | 96.09 | 33 |
| 16-F23 | 10470666 | 100 | 97.59 | 28 |
| 16-F24 | 8987040 | 100 | 97.04 | 24 |
| 16-F25 | 11928975 | 100 | 96.69 | 31 |
| 16-F26 | 10870741 | 100 | 96.64 | 28 |
| 16-F27 | 11647159 | 100 | 96.38 | 30 |
| 16-F28 | 11051769 | 100 | 95.63 | 29 |
| 16-F29 | 12977620 | 100 | 95.68 | 34 |
| 16-F30 | 11117965 | 100 | 96.37 | 29 |
| 16-F31 | 12540975 | 100 | 95.32 | 32 |
| 16-F32 | 14742865 | 100 | 96.24 | 38 |
| 16-F33 | 13308868 | 100 | 95.06 | 34 |
| 16-F34 | 14109197 | 100 | 96.54 | 37 |
| 16-F35 | 13761148 | 100 | 95.91 | 36 |
| 16-F36 | 11071775 | 100 | 96.74 | 29 |
| 16-F37 | 12359187 | 100 | 97.07 | 32 |
| 16-F38 | 11481667 | 100 | 96.57 | 30 |
| 16-F39 | 12845661 | 100 | 96.18 | 33 |
| 16-F40 | 10745460 | 100 | 98.09 | 28 |
| 16-F41 | 10921792 | 100 | 97.72 | 29 |

Table 2.2. Genome sequencing statistics for all 190 *C. beticola* isolates in this study (continued).

| Isolate Name | Total number of reads | Sequence length (bp) | Reference genome alignment rate (%) | Mean reference genome coverage (X) |
|---|---|---|---|---|
| 16-F42 | 12209843 | 100 | 96.34 | 32 |
| 16-F43 | 13446151 | 100 | 96.2 | 35 |
| 16-F44 | 12746723 | 100 | 97.21 | 33 |
| 16-F45 | 10878158 | 100 | 96.03 | 28 |
| 16-F46 | 12654252 | 100 | 97.06 | 33 |
| 16-F47 | 11235283 | 100 | 96.2 | 29 |
| 16-F48 | 9994376 | 100 | 96.07 | 26 |
| 16-F49 | 11624916 | 100 | 96.49 | 30 |
| 16-F50 | 12212953 | 100 | 97.35 | 32 |
| 16-F51 | 12106448 | 100 | 96.02 | 31 |
| 16-F52 | 11021092 | 100 | 97.42 | 29 |
| 16-F53 | 12586280 | 100 | 95.94 | 33 |
| 16-F54 | 10207809 | 100 | 96 | 26 |
| 16-F55 | 9733604 | 100 | 96.6 | 25 |
| 16-F56 | 12880745 | 100 | 97.14 | 34 |
| 16-F57 | 13340824 | 100 | 97.4 | 35 |
| 16-F58 | 10964146 | 100 | 95.33 | 28 |
| 16-F59 | 11398428 | 100 | 95.98 | 30 |
| 16-16 | 15393529 | 100 | 95.45 | 40 |
| 16-45 | 15338757 | 100 | 95.17 | 39 |
| 16-46 | 15337957 | 100 | 94.76 | 39 |
| 16-62 | 15248588 | 100 | 94.95 | 39 |
| 16-92 | 15075186 | 100 | 90.35 | 37 |
| 16-131 | 15179611 | 100 | 94.25 | 39 |
| 16-175 | 13872122 | 100 | 95.74 | 36 |
| 16-339 | 12810204 | 100 | 95.31 | 33 |
| 16-591 | 10930000 | 100 | 95.98 | 28 |
| 16-665 | 12737134 | 100 | 95.95 | 33 |
| 16-735 | 12459059 | 100 | 96.02 | 32 |
| 16-781 | 10895348 | 100 | 95.99 | 28 |
| 16-787 | 10523070 | 100 | 96.08 | 27 |
| 16-805 | 12611611 | 100 | 95.92 | 33 |
| 16-810 | 9525664 | 100 | 95.79 | 25 |
| 16-890 | 11498111 | 100 | 96.39 | 30 |
| 16-1148 | 10304038 | 100 | 95.94 | 27 |
| 16-26 | 13359646 | 100 | 95.09 | 34 |
| 16-33 | 13508108 | 100 | 95.67 | 35 |
| 16-72 | 13793982 | 100 | 94.15 | 35 |
| 16-93 | 13455330 | 100 | 93.24 | 34 |

Table 2.2. Genome sequencing statistics for all 190 *C. beticola* isolates in this study (continued).

| Isolate Name | Total number of reads | Sequence length (bp) | Reference genome alignment rate (%) | Mean reference genome coverage (X) |
|---|---|---|---|---|
| 16-317 | 13515885 | 100 | 95.53 | 35 |
| 16-414 | 13338997 | 100 | 94.32 | 34 |
| 16-498 | 13530538 | 100 | 94.87 | 35 |
| 16-523 | 13478816 | 100 | 95.15 | 35 |
| 16-665 | 13418499 | 100 | 95.07 | 34 |
| 16-704 | 13727960 | 100 | 94.66 | 35 |
| 16-716 | 15463661 | 100 | 92.43 | 39 |
| 16-722 | 15239636 | 100 | 95.86 | 39 |
| 16-724 | 15323018 | 100 | 95.85 | 40 |
| 16-790 | 15338450 | 100 | 96.02 | 40 |
| 16-819 | 15289810 | 100 | 96.14 | 40 |
| 16-868 | 15338708 | 100 | 95.81 | 40 |
| 16-1105 | 15381554 | 100 | 95.88 | 40 |
| 16-1169 | 15320524 | 100 | 95.25 | 39 |
| 16-F61 | 8889309 | 150 | 96.35 | 35 |
| 16-F62 | 8931302 | 150 | 96.21 | 35 |
| 16-F63 | 8922341 | 150 | 96.72 | 35 |
| 16-F64 | 8929826 | 150 | 96.04 | 35 |
| 16-F65 | 8170519 | 150 | 95.83 | 32 |
| 16-100 | 8154726 | 150 | 96.03 | 32 |
| 16-1022 | 8387188 | 150 | 95.48 | 32 |
| 16-1083 | 8181686 | 150 | 94.99 | 31 |
| 16-1110 | 8322394 | 150 | 96.25 | 32 |
| 16-1124 | 8213048 | 150 | 95.8 | 32 |
| 16-1129 | 8228243 | 150 | 96.2 | 32 |
| 16-1138 | 8229474 | 150 | 95.84 | 32 |
| 16-1151 | 8021108 | 150 | 95.8 | 31 |
| 16-1155 | 8249103 | 150 | 96.73 | 32 |
| 16-1211 | 8204928 | 150 | 96.05 | 32 |
| 16-1214 | 8233643 | 150 | 95.79 | 32 |
| 16-123 | 8141259 | 150 | 96 | 32 |
| 16-141 | 8217388 | 150 | 96.3 | 32 |
| 16-163 | 8113850 | 150 | 96.36 | 32 |
| 16-1044 | 7390194 | 150 | 95.91 | 29 |
| 16-181 | 8203241 | 150 | 96.45 | 32 |
| 16-186 | 8190961 | 150 | 96.11 | 32 |
| 16-224 | 7214579 | 150 | 95.53 | 28 |
| 16-270 | 8360024 | 150 | 96.19 | 33 |
| 16-29 | 8038226 | 150 | 96.29 | 31 |

Table 2.2. Genome sequencing statistics for all 190 *C. beticola* isolates in this study (continued).

| Isolate Name | Total number of reads | Sequence length (bp) | Reference genome alignment rate (%) | Mean reference genome coverage (X) |
|---|---|---|---|---|
| 16-326 | 8025845 | 150 | 96.07 | 31 |
| 16-351 | 8064410 | 150 | 96.25 | 31 |
| 16-36 | 8079058 | 150 | 96.04 | 31 |
| 16-363 | 8075125 | 150 | 96.38 | 32 |
| 16-374 | 7468268 | 150 | 94.99 | 29 |
| 16-412 | 8153998 | 150 | 96.12 | 32 |
| 16-438 | 8150620 | 150 | 96.01 | 32 |
| 16-451 | 8233113 | 150 | 96.25 | 32 |
| 16-503 | 8181538 | 150 | 95.36 | 32 |
| 16-54 | 8047244 | 150 | 95.97 | 31 |
| 16-546 | 8567009 | 150 | 62.02 | 22 |
| 16-569 | 8223639 | 150 | 95.99 | 32 |
| 16-638 | 8242271 | 150 | 95.25 | 32 |
| 16-723 | 8288548 | 150 | 96.3 | 32 |
| 16-731 | 8181859 | 150 | 96 | 32 |
| 16-795 | 8252996 | 150 | 96.61 | 32 |
| 16-827 | 8230298 | 150 | 96.33 | 32 |
| 16-842 | 8269336 | 150 | 96.48 | 32 |
| 16-879 | 8239261 | 150 | 97.09 | 32 |
| 16-90 | 8233696 | 150 | 96.57 | 32 |
| 16-938 | 7329161 | 150 | 96 | 28 |
| 16-946 | 8281553 | 150 | 96.83 | 32 |
| 16-987 | 8325224 | 150 | 96.44 | 32 |
| 17-1015 | 8224169 | 150 | 96.73 | 32 |
| 17-1045 | 8214141 | 150 | 96.48 | 32 |
| 17-1047 | 8166920 | 150 | 95.85 | 32 |
| 17-1070 | 8247959 | 150 | 96.21 | 32 |
| 17-1096 | 8671766 | 150 | 51.31 | 18 |
| 17-1109 | 8255032 | 150 | 96.31 | 32 |
| 17-1121 | 8183028 | 150 | 96.5 | 32 |
| 17-1127 | 8240663 | 150 | 96.2 | 32 |
| 17-1132 | 7646731 | 150 | 95.88 | 30 |
| 17-1162 | 8272871 | 150 | 96.92 | 32 |
| 17-132 | 8195993 | 150 | 96.39 | 32 |
| 17-177 | 8146135 | 150 | 96.37 | 32 |
| 17-214 | 8255259 | 150 | 96.84 | 32 |
| 17-235 | 8300163 | 150 | 96.46 | 32 |
| 17-251 | 8254369 | 150 | 96.46 | 32 |
| 17-300 | 8213705 | 150 | 97 | 32 |

Table 2.2. Genome sequencing statistics for all 190 *C. beticola* isolates in this study (continued).

| Isolate Name | Total number of reads | Sequence length (bp) | Reference genome alignment rate (%) | Mean reference genome coverage (X) |
|---|---|---|---|---|
| 17-307 | 8192075 | 150 | 96.58 | 32 |
| 17-354 | 8186482 | 150 | 96.66 | 32 |
| 17-363 | 8200985 | 150 | 96.58 | 32 |
| 17-385 | 8165428 | 150 | 95.39 | 32 |
| 17-390 | 8246570 | 150 | 96.42 | 32 |
| 17-410 | 8235966 | 150 | 96.51 | 32 |
| 17-443 | 8231728 | 150 | 96.62 | 32 |
| 17-478 | 8223309 | 150 | 96.57 | 32 |
| 17-48 | 8208937 | 150 | 96.65 | 32 |
| 17-51 | 8291290 | 150 | 96.44 | 32 |
| 17-516 | 8198392 | 150 | 96.74 | 32 |
| 17-528 | 8169356 | 150 | 97.23 | 32 |
| 17-585 | 8211420 | 150 | 96.59 | 32 |
| 17-594 | 8250461 | 150 | 97.4 | 33 |
| 17-633 | 8214609 | 150 | 96.88 | 32 |
| 17-661 | 8375374 | 150 | 96.36 | 33 |
| 17-696 | 8229868 | 150 | 96.61 | 32 |
| 17-724 | 8320330 | 150 | 96.34 | 32 |
| 17-741 | 8165723 | 150 | 96.58 | 32 |
| 17-760 | 8307505 | 150 | 96.76 | 33 |
| 17-799 | 8306128 | 150 | 96.4 | 32 |
| 17-82 | 8153198 | 150 | 96.63 | 32 |
| 17-837 | 8412166 | 150 | 96.97 | 33 |
| 17-852 | 8252594 | 150 | 96.38 | 32 |
| 17-869 | 8332658 | 150 | 96.75 | 33 |
| 17-912 | 8363854 | 150 | 96.7 | 33 |
| 17-933 | 8290847 | 150 | 96.86 | 33 |
| 16-1170 | 8267245 | 150 | 96.54 | 32 |
| 16-892 | 8184082 | 150 | 96.5 | 32 |
| 17-1087 | 8215639 | 150 | 96.84 | 32 |
| 17-256 | 8297680 | 150 | 96.04 | 32 |
| 17-432 | 8236108 | 150 | 96.53 | 32 |
| 17-501 | 8200429 | 150 | 96.78 | 32 |
| 17-950 | 8291920 | 150 | 96.74 | 32 |

Mapping power of GWAS was assessed by calculating linkage disequilibrium (LD) decay for the population. LD decayed to $r^2 < 0.2$ rapidly within ~3.5 kb (Fig. 2.1), which is comparable to values found in populations of other closely-related filamentous fungal phytopathogens used successfully for GWAS such as *Zymoseptoria tritici* (Hartmann et al. 2017) and *Parastagonospora nodorum* (Gao et al. 2016; Pereira et al. 2020; Richards et al. 2019).

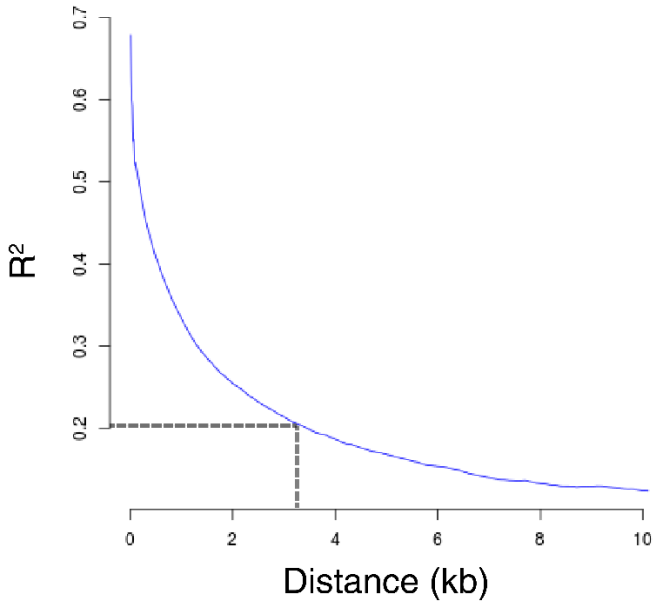


Figure 2.1. Linkage disequilibrium (LD) decay in *C. beticola*. LD decay was calculated as squared correlation of allele frequencies ($R^2$) between all pairwise combinations of markers within 10kb of each other in the population of 190 *C. beticola* isolates. LD decays to 0.2 within 3.5kb.

### 2.4.3. Population structure analyses

We performed a principle component analysis (PCA) to visually assess population structure amongst the 190 *C. beticola* isolates. PC1 explained 11% of total variation followed by 3.4% and 3.0% for PCs 2 and 3, respectively. Analysis of population structure often reveals clustering of individuals according to geographical origin. However, pairwise plots of the first two PCs from PCA demonstrated that sampling location had little impact on clustering of the *C. beticola* isolates used in this study (Fig. 2.2). Intriguingly, the tight cluster of strains seen in

Figure 2.2 are predominantly tetraconazole-sensitive isolates, whereas the remaining scattered isolates are mainly tetraconazole-resistant isolates. This illustrates how fungicide use is shaping population structure in RRV *C. beticola*.

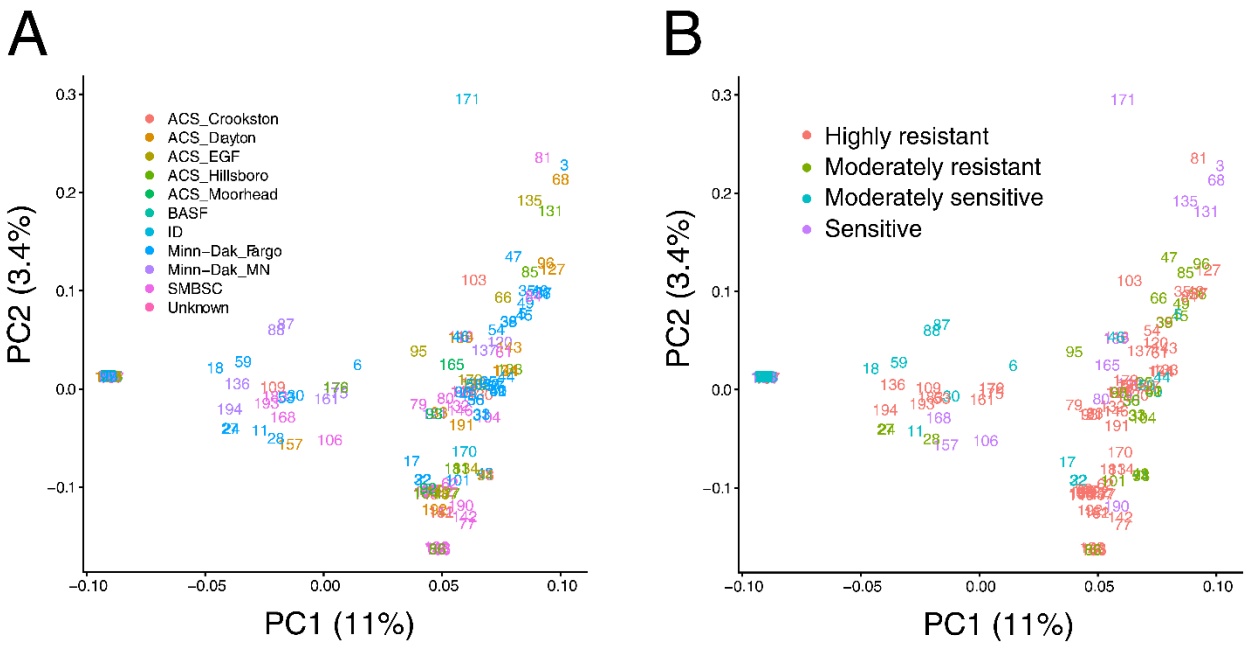


Figure 2.2. Principle component analyses. The first two principle components plotted from a PCA of *C. beticola* isolates performed with 37,973 LD-pruned genome-wide SNPs. Plots are color-coded by A) field sampling location and B) tetraconazole sensitivity. Highly resistant = isolates with $EC_{50} \geq 10\mu g/mL$; Moderately resistant = isolates $1 \leq EC_{50} < 10$; Moderately sensitive = isolates with $0.1 \leq EC_{50} < 1$; Sensitive = isolates with $EC_{50} < 0.1$.

### 2.4.4. Genetic architecture of tetraconazole sensitivity

To determine the genetic architecture of tetraconazole sensitivity in *C. beticola*, we performed GWAS using all 190 isolates. Model selection was based on visualization of Q-Q plots to ensure correction for population structure, as well as the use of GAPIT Bayesian Information Criterion to determine the optimal number of principle components (zero in this case). We also looked for the most significantly associated markers to consistently appear throughout multiple models. The MLM model chosen yielded thirteen significant associations at a significance threshold of $-\log_{10}(P\text{-value}) = 4.5$ (Fig. 2.3, Table 2.3, Fig. 2.4). Of these

associated markers, ten were intragenic SNPs, two were indels within 5'UTRs and one was within 124bp of the nearest gene.

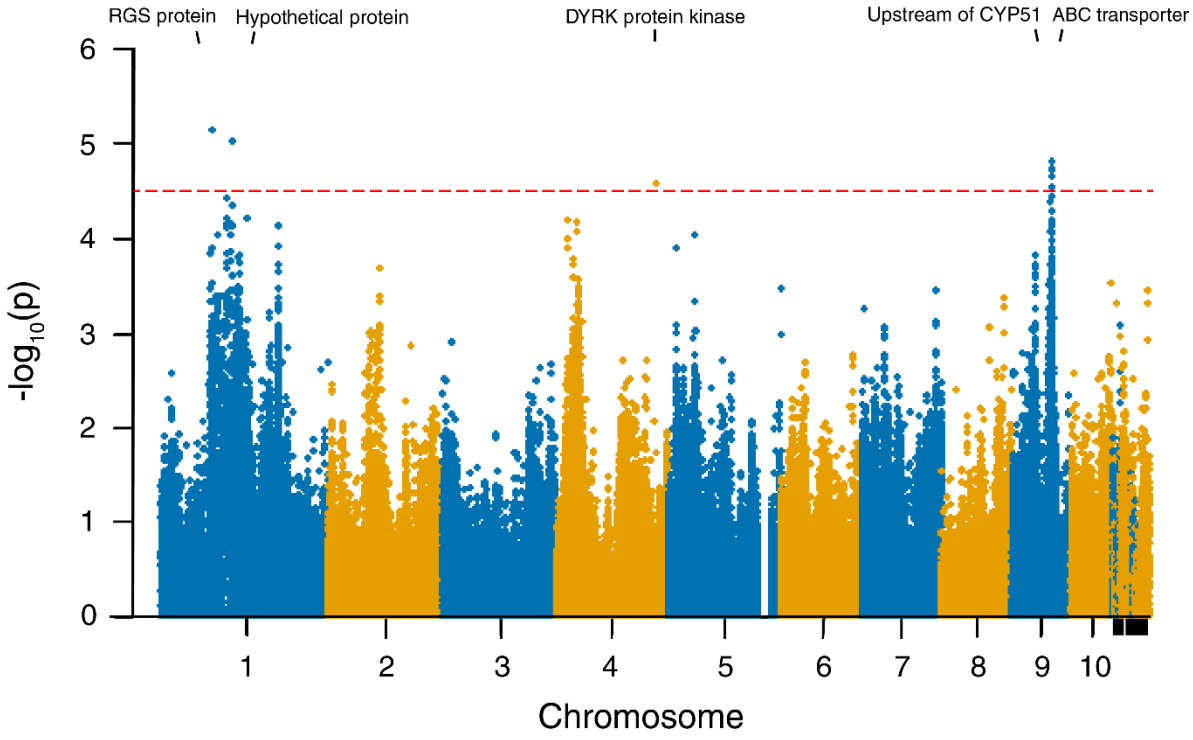


Figure 2.3. GWAS of tetraconazole sensitivity in *C. beticola.* Manhattan plot displaying marker associations with tetraconazole $EC_{50}$ values. The red line represents the significance threshold of $-\log_{10}(P)=4.5$. The genomic position of genes with significantly associated markers are indicated above the plot.

Table 2.3. Thirteen significant associations for GWAS of tetraconazole sensitivity at a significance threshold of -$\log_{10}(p)$ = 4.5.

| Chr[1] | Position (bp) | *P*-value | -log10(*P*) | Reference allele | Alternate allele | Gene ID | Gene annotation | Mutation | MAF[2] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1890198 | 7.04E-06 | 5.1524 | C | - | CB0940_00689 | Sorting nexin-12 | 5'UTR 76bp indel | 0.27 |
| 1 | 2637787 | 9.41E-06 | 5.0264 | A | G | CB0940_00999 | Hypothetical protein | S474G | 0.47 |
| 9 | 1503309 | 1.56E-05 | 4.8069 | C | G | CB0940_11399 | ABC transporter CDR4 | Synonymous (L191) | 0.47 |
| 9 | 1452111 | 1.83E-05 | 4.7375 | A | G | - | - | Intergenic | 0.47 |
| 9 | 1503297 | 1.84E-05 | 4.7352 | A | C | CB0940_11399 | ABC transporter CDR4 | Synonymous (L195) | 0.47 |
| 9 | 1503321 | 1.95E-05 | 4.7100 | A | T | CB0940_11399 | ABC transporter CDR4 | Synonymous (G187) | 0.47 |
| 9 | 1503303 | 1.95E-05 | 4.7100 | T | C | CB0940_11399 | ABC transporter CDR4 | Synonymous (K193) | 0.46 |
| 9 | 1503315 | 1.96E-05 | 4.7077 | G | A | CB0940_11399 | ABC transporter CDR4 | Synonymous (S189) | 0.47 |
| 9 | 1503312 | 1.96E-05 | 4.7077 | C | G | CB0940_11399 | ABC transporter CDR4 | Synonymous (T190) | 0.47 |
| 9 | 1502965 | 2.21E-05 | 4.6556 | T | G | CB0940_11399 | ABC transporter CDR4 | Intron | 0.48 |
| 4 | 3725552 | 2.59E-05 | 4.5867 | A | - | CB0940_05141 | Dual specificity protein kinase Pom1 | 5'UTR 63bp indel | 0.23 |
| 9 | 1502968 | 2.88E-05 | 4.5406 | G | C | CB0940_11399 | ABC transporter CDR4 | Intron | 0.47 |
| 9 | 1502966 | 2.88E-05 | 4.5406 | C | T | CB0940_11399 | ABC transporter CDR4 | Intron | 0.47 |

[1]Chromosome/scaffold number

[2]Minor allele frequency

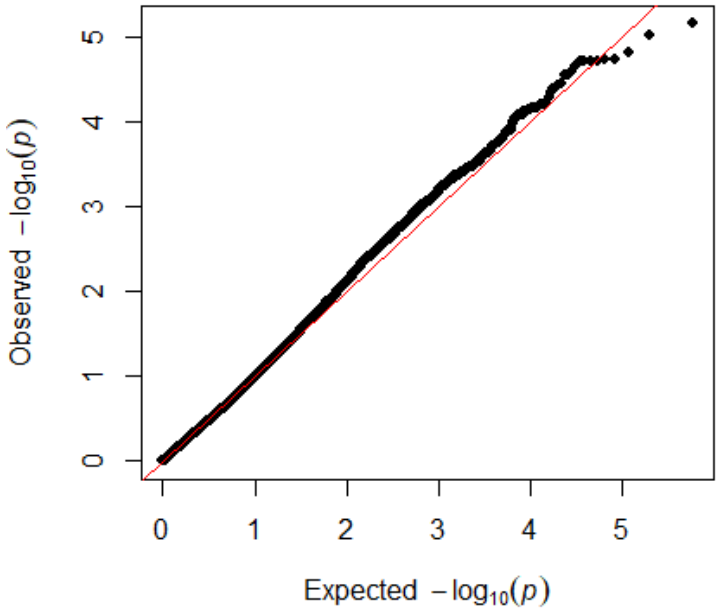


Figure 2.4. Quantile-Quantile plot for genome-wide association of tetraconazole sensitivity.

*2.4.4.1. RGS domain protein*

The most significantly associated marker was 1_1890198 which is within a 76bp indel in the 5'UTR of gene CB0940_00689 encoding a protein with a regulator of G-protein signalling (RGS) domain. The reference genome harbors the insertion (spanning 1,890,172-1,890,247bp), which is absent in the alternate allele. The 5'UTR insertion is associated with a significant increase in tetraconazole $EC_{50}$ value ($P < 0.01$) (Fig. 2.5A). The predicted 1,232 amino acid sequence has multiple conserved domains including a PXA domain, a flagellar switch protein FliM domain, a regulator of G-protein signalling (RGS) domain, the phosphoinositide binding Phox homology (PX) domain of yeast Mdm1 and a C-terminus sorting nexin domain.

*2.4.4.2. Hypothetical protein*

The second most significantly associated marker was the SNP 1_2637787 within hypothetical protein gene CB0940_00999 which lacks domains of known function. The SNP confers an amino acid substitution from glycine to serine at codon 474 (G474S) out of 488 amino

acids. The serine substitution (due to A reference allele) gives rise to a significant increase in tetraconazole $EC_{50}$ value ($P < 0.001$) ($P < 0.01$) (Fig. 2.5B).

*2.4.4.3. ABC PDR transporter*

The third most significant association was SNP 9_1503309 within the ATP-binding cassette (ABC) transporter gene CB0940_11399. This mutation confers a synonymous substitution at codon 191 (L191). The C allele was associated with a significant increase in tetraconazole $EC_{50}$ when compared to the G allele ($P < 0.001$) (Fig. 2.5C). Eight additional SNPs within this gene were significantly associated with tetraconazole sensitivity, five of these were synonymous mutations (at codons G187, S189, T190, K193 and L195) in the first exon and three were within the second intron (Table 2.3). This protein sequence is comprised of 1,493 amino acids with a highly conserved ABC-2 transporter domain and pleiotropic drug resistance (PDR) sub-family nucleotide-binding domain. In total, 135 polymorphisms were identified within the predicted gene sequence. The majority are found at the 5' end of the gene, with 82 located within the first exon (the first 726 bp of 4,482 bp coding sequence). Of these, six SNPs in complete LD with the most significantly associated SNP 9_1503309 that give rise to amino acid substitutions: E29D, R33Q, H47R, D152E, I210V and N218S. There are no indels immediately upstream of the start codon, but there are multiple SNPs in high LD with the associated mutations.

*2.4.4.4. Upstream of CYP51*

The fourth most significant association was SNP 9_1452111 and lies ~50kb upstream of the ABC transporter gene on chromosome nine. This SNP is just 124bp upstream of the start codon of DMI fungicide target gene *CYP51*. When A is present instead of G, there is a significant increase in tetraconazole $EC_{50}$ value ($P < 0.001$) (Fig. 2.5D).

*2.4.4.5. Dual-specificity DYRK protein kinase*

The fifth most significantly associated locus was marker 4_3725552. This is an indel within the 5'UTR of a putative dual specificity protein kinase (gene CB0940_05141). The 61bp insertion in the reference allele is associated with an increase in tetraconazole $EC_{50}$ value ($P <$ 0.001) (Fig. 2.5E). This 263-amino acid protein has a highly conserved dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) domain that enables the kinase to phosphorylate serine/threonine residues in other proteins and autophosphorylate via tyrosine residues.

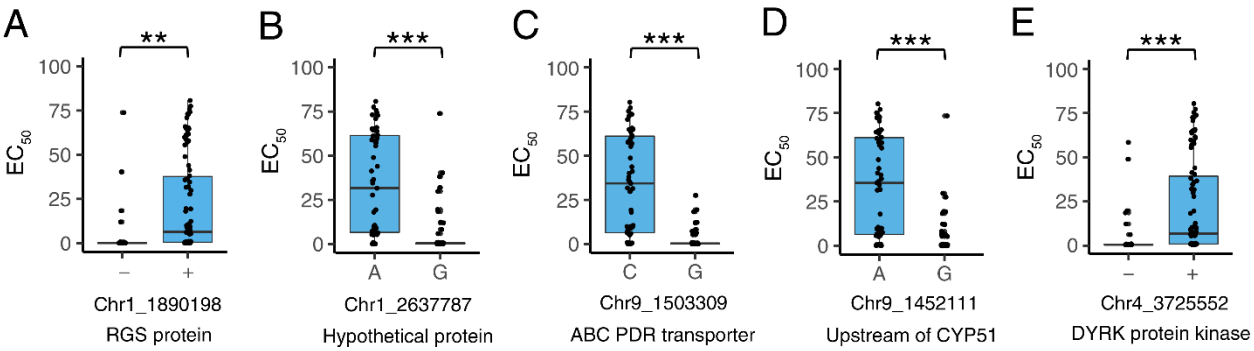


Figure 2.5. Effects of significantly associated markers on tetraconazole sensitivity. The effects of DMI fungicide resistance loci A) indel 1_1890198 in the 5'UTR of a RGS protein gene, B) SNP 1_2637787 within a hypothetical protein gene, C) SNP 9_1503309 within an ABC PDR transporter gene, D) SNP 9_1452111 upstream of *CYP51* and E) indel 4_3725552 in the 5'UTR of a DYRK kinase gene. Significant differences in $EC_{50}$ between alleles are displayed at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

### 2.4.5. *CYP51* haplotype analysis

Genome-wide association analyses of tetraconazole sensitivity suggested the involvement of the *CYP51* locus, with a significantly associated SNP 9_1452111 124bp upstream of the *CYP51* start codon (Fig. 2.3). In *C. beticola*, *CYP51* is a single copy intron-free gene of 1,632 bp (NCBI XP_023450255.1) (Bolton et al. 2012b; Nikou et al. 2009). Since intrinsic *CYP51* expression was previously shown to be higher in some resistant *C. beticola* strains (Bolton et al. 2012b; Bolton et al. 2016; Nikou et al. 2009), we questioned whether mutations upstream of *CYP51* may affect *CYP51* expression and fungicide resistance. No

insertions or retrotransposons were found upstream of *CYP51*, only several SNPs such as 9_1452111 within 3kb either side of the gene. Variation in read depth was not observed at the *CYP51* locus, suggesting no variation in copy number. The SNP 9_1452111 is notably in high LD with a silent mutation at E170 ($R^2 = 0.7$), thus exhibiting similar effects on $EC_{50}$ value. All isolates with E170 also had the upstream SNP. Eighteen isolates harbored the upstream SNP without E170, but these also had non-synonymous changes in CYP51 (not shown). Therefore, we cannot evaluate the individual effects of the upstream SNP and E170 on tetraconazole $EC_{50}$ value without functional studies.

The putative effects of the 9_1452111 SNP upstream of *CYP51* on transcription factor binding sites were predicted using the JASPAR database (Fornes et al. 2020) (Table 2.4). Query sequences of 21bp revealed differences in putative binding sites (all defined experimentally in *Saccharomyces cerevisiae*) for both versions of the SNP (Table 2.4). UPC2 and STE12 bindings sites were present for both versions, but the resistant allele (T) had a stronger UPC2 binding site predicted on the negative strand and a weaker STE12 binding site predicted on the positive strand. The resistant allele (T) also had GLN3 and SPT23 binding sites predicted that were absent for the sensitive allele (C). The sensitive allele (C) had ECM22 and STB5 bindings sites uniquely predicted.

Table 2.4. Predicted transcription factor binding sites for the 9_1452111 SNP upstream of *CYP51* using the JASPAR database.

| Sequence ID[1] | Matrix ID[2] | TF Name[3] | Score[4] | Relative score[5] | Start (bp)[6] | End (bp)[6] | Strand[7] | Predicted sequence[8] |
|---|---|---|---|---|---|---|---|---|
| Sensitive_C | MA0393.1 | STE12 | 10.00 | 0.96 | 6 | 12 | + | tgaaacc |
| Sensitive_C | MA0411.1 | UPC2 | 8.70 | 0.94 | 12 | 18 | + | cgtacga |
| Sensitive_C | MA0411.1 | UPC2 | 5.80 | 0.84 | 11 | 17 | - | cgtacgg |
| Sensitive_C | MA0292.1 | ECM22 | 5.98 | 0.84 | 9 | 15 | + | aaccgta |
| Sensitive_C | MA0392.1 | STB5 | 6.32 | 0.84 | 6 | 13 | - | cggtttca |
| Resistant_T | MA0411.1 | UPC2 | 8.70 | 0.94 | 11 | 17 | - | cgtacga |
| Resistant_T | MA0411.1 | UPC2 | 8.70 | 0.94 | 12 | 18 | + | cgtacga |
| Resistant_T | MA0307.1 | GLN3 | 4.82 | 0.86 | 8 | 12 | - | gattt |
| Resistant_T | MA0393.1 | STE12 | 2.35 | 0.83 | 6 | 12 | + | tgaaatc |
| Resistant_T | MA0388.1 | SPT23 | 5.00 | 0.82 | 5 | 12 | - | gatttcaa |
| Resistant_T | MA0388.1 | SPT23 | 4.20 | 0.80 | 7 | 14 | + | gaaatcgt |
| Resistant_T | MA0388.1 | SPT23 | 4.11 | 0.80 | 8 | 15 | + | aaatcgta |

[1]Sequences of 21bp in length were used as queries with the 11$^{th}$ bp as the sensitive C allele or the resistant T allele.
[2]Position frequency matrices in JASPAR database representing DNA-binding sites determined experimentally for a transcription factor and used to scan query sequences.
[3]Name of transcription factor in *Saccharomyces cerevisiae*.
[4]Raw score for binding site.
[5]Relative score is the fraction of the potential maximum score for the binding site.
[6]The predicted start and end base pairs of the binding site within the query sequence.
[7]Binding site is present on either the positive (forward) or negative (reverse) strand of DNA.
[8]Predicted binding site within query sequence.

We also investigated the presence of target site mutations in CYP51 that may influence DMI sensitivity. We found 11 different *CYP51* gene coding sequences (haplotypes) in our set of 190 RRV region isolates. There were two different "DMI-sensitive" haplotypes; the most common harbored by 85 isolates (haplotype 3) and another highly divergent haplotype (haplotype 1) found in three isolates that harbored 113 SNPs and an 82 bp deletion (Fig. 2.6). Additionally, haplotype 2 was represented by a single sensitive isolate harboring a silent mutation at I122 and the amino acid substitution V467A (Fig. 2.6). When comparing the remaining haplotypes to the most common sensitive haplotype (#3), there were five different non-synonymous mutations (L144F, I309T, I387M, Y464S and V467A; Fig. 2.6). The presence of amino acid substitutions L144F, I387M or Y464S gave higher tetraconazole $EC_{50}$ values when compared to sensitive haplotype #2 (Fig. 2.6).

| Haplotype | Mutations | Count |
|---|---|---|
| 1 | Sensitive allele #1 | 3 |
| 2 | I122 (silent) + V467A | 1 |
| 3 | Sensitive allele #2 | 85 |
| 4 | L144F (T) | 18 |
| 5 | L144F (T) + E170 (silent) | 2 |
| 6 | E170 (silent) | 56 |
| 7 | L144F (C) + E170 (silent) | 12 |
| 8 | L144F (C) + E170 (silent) + I309T | 2 |
| 9 | I387M | 2 |
| 10 | L144F (C) | 7 |
| 11 | Y464S | 2 |

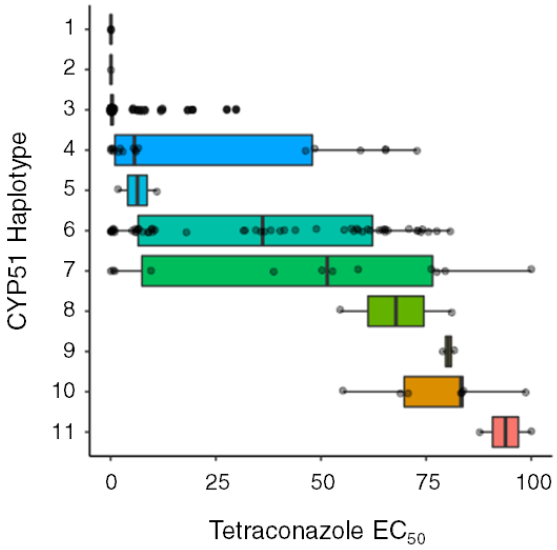


Figure 2.6. Effects of *CYP51* haplotypes on tetraconazole sensitivity. The left panel displays the 11 different *CYP51* coding sequence haplotypes found in our *C. beticola* population with the respective number of isolates with each haplotype. Mutations were identified as compared to the most common sensitive Haplotype (#3). The right panel displays box and whiskers plots for tetraconazole EC50 values for each *CYP51* haplotype.

The most common amino acid substitution found was L144F (41 isolates) and could be achieved by either a T or C mutation in the 3rd position of the 144th codon TTG (Fig. 2.6, 2.7, 2.8). Both TTT and TTC versions of L144F were associated with increased tetraconazole $EC_{50}$ values ($P < 0.01$ and $P < 0.001$, respectively), but the TTC version had a significantly higher mean $EC_{50}$ value than the TTT version ($P < 0.001$) (Fig. 2.7). Since synonymous codons at position 144 were associated with differential tetraconazole resistance, we questioned whether codon bias might help explain this phenomenon. Consequently, we calculated genome-wide codon usage for *C. beticola* (Table 2.5). The phenylalanine codon TTC is found in coding sequence 70% of the time compared to TTT, which is found in the remaining 30%. This is the largest difference in codon usage for any amino acid found in *C. beticola*.

The most common *CYP51* haplotype associated with resistance (56 isolates) had a single silent mutation at E170 changing the 170th codon from GAG to GAA, both of which encode glutamic acid (Fig. 2.6). Presence of the E170 mutation was associated with a significant increase in tetraconazole $EC_{50}$ value ($P < 0.001$, Fig. 2.7). Since codon usage again could underscore DMI resistance, we assessed codon frequencies for glutamic acid (E) and found that the GAG codon is used slightly more often (56%) than the GAA codon (44%) (Table 2.5).

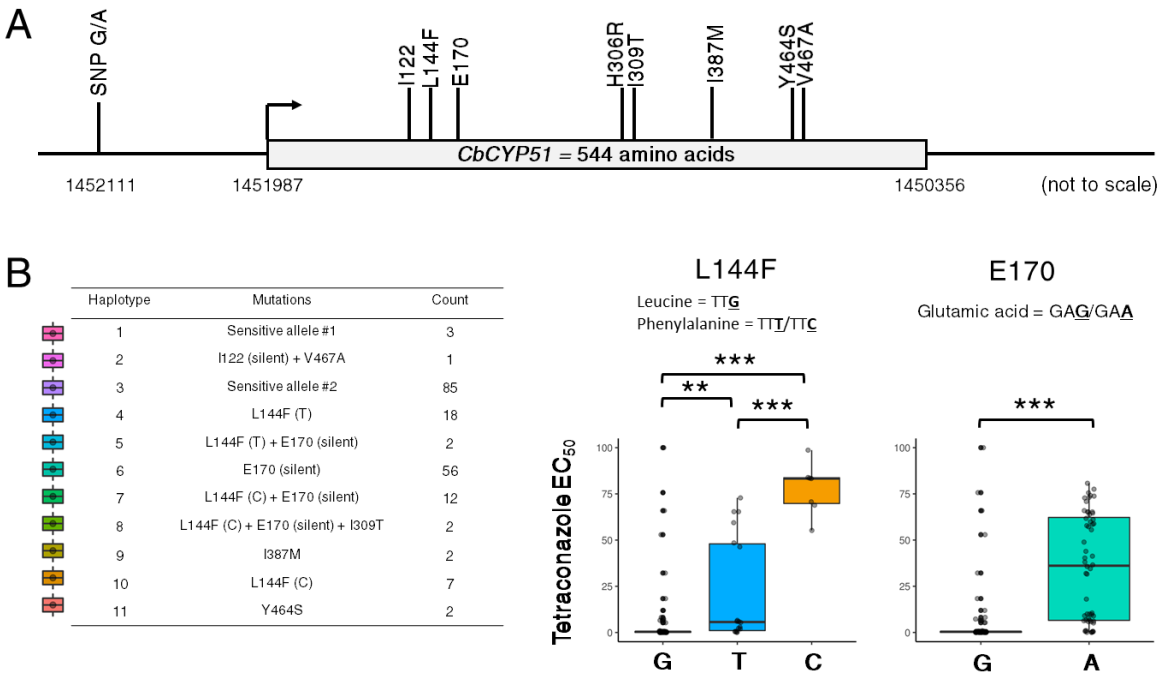


Figure 2.7. *CYP51* gene model in *C. beticola* and the effects of L144F and E170 mutations on tetraconazole $EC_{50}$ value. A) *CYP51* gene model displaying all coding mutations found in this study, and a single upstream mutation. B) Isolates with single *CYP51* mutations were used to compare the effects of L144F codons; phenyalanine codons TTT and TTC both gave significantly higher $EC_{50}$ values than leucine codon TTG ($P < 0.01$), but codon TTC also gave significantly higher $EC_{50}$ values than codon TTT ($P < 0.001$). Isolates with single *CYP51* mutations were also used to compare the effects of E170; glutamic acid codon GAA gave significantly higher $EC_{50}$ values than codon GAG ($P < 0.001$).

Table 2.5. Codon usage for the *C. beticola* 09-40 reference genome.

| Amino acid | Codon | Total number | Proportion of amino acid codon usage (%) |
|---|---|---|---|
| Ala | GCG | 118196 | 22 |
| Ala | GCA | 145227 | 27 |
| Ala | GCT | 140842 | 26 |
| Ala | GCC | 142178 | 26 |
| Cys | TGT | 33364 | 37 |
| Cys | TGC | 56751 | 63 |
| Asp | GAT | 161658 | 47 |
| Asp | GAC | 179197 | 53 |
| Glu | GAG | 209892 | 56 |
| Glu | GAA | 162472 | 44 |
| Phe | TTT | 62313 | 30 |
| Phe | TTC | 147005 | 70 |
| Gly | GGG | 53396 | 13 |
| Gly | GGA | 111288 | 26 |
| Gly | GGT | 90404 | 21 |
| Gly | GGC | 166563 | 40 |
| His | CAT | 72845 | 46 |
| His | CAC | 87140 | 54 |
| Ile | ATA | 35549 | 13 |
| Ile | ATT | 86957 | 32 |
| Ile | ATC | 150516 | 55 |
| Lys | AAG | 187064 | 66 |
| Lys | AAA | 98367 | 34 |
| Leu | TTG | 97005 | 19 |
| Leu | TTA | 20659 | 4 |
| Leu | CTG | 126837 | 25 |
| Leu | CTA | 41126 | 8 |
| Leu | CTT | 85003 | 17 |
| Leu | CTC | 141355 | 28 |
| Met | ATG | 123264 | 100 |
| Asn | AAT | 89810 | 42 |
| Asn | AAC | 126518 | 58 |
| Pro | CCG | 81410 | 23 |
| Pro | CCA | 112773 | 32 |
| Pro | CCT | 91854 | 26 |
| Pro | CCC | 70565 | 20 |
| Gln | CAG | 140058 | 52 |
| Gln | CAA | 128544 | 48 |
| Arg | AGG | 48644 | 12 |

Table 2.5. Codon usage for the *C. beticola* 09-40 reference genome (continued).

| Amino acid | Codon | Total number | Proportion of amino acid codon usage (%) |
|---|---|---|---|
| Arg | AGA | 65561 | 16 |
| Arg | CGG | 47562 | 12 |
| Arg | CGA | 96019 | 23 |
| Arg | CGT | 56083 | 14 |
| Arg | CGC | 98064 | 24 |
| Ser | AGT | 62408 | 13 |
| Ser | AGC | 111131 | 23 |
| Ser | TCG | 87936 | 18 |
| Ser | TCA | 70090 | 14 |
| Ser | TCT | 76449 | 16 |
| Ser | TCC | 81223 | 17 |
| Thr | ACG | 86442 | 24 |
| Thr | ACA | 94404 | 26 |
| Thr | ACT | 83744 | 23 |
| Thr | ACC | 96140 | 27 |
| Val | GTG | 106502 | 30 |
| Val | GTA | 41238 | 12 |
| Val | GTT | 70894 | 20 |
| Val | GTC | 132150 | 38 |
| Trp | TGG | 92885 | 100 |
| Tyr | TAT | 61128 | 38 |
| Tyr | TAC | 101542 | 62 |
| End | TGA | 18264 | 62 |
| End | TAG | 5995 | 20 |
| End | TAA | 5048 | 17 |

To further investigate the involvement of these mutations in DMI fungicide resistance, we sequenced *CYP51* in 52 additional *C. beticola* isolates obtained from the Red River Valley of North Dakota and Minnesota in 2019. The results largely corroborated the findings from *CYP51* screening of 2016 and 2017 GWAS isolates. As before, the most common haplotype associated with resistance had the silent mutation E170 (Fig. 2.8). We found that the amino acid substitutions L144F and Y464S were again associated with increased tetraconazole $EC_{50}$ values (Fig. 2.8). The amino acid mutation H306R was also found alongside L144F in a single 2019 isolate (Fig. 2.8).

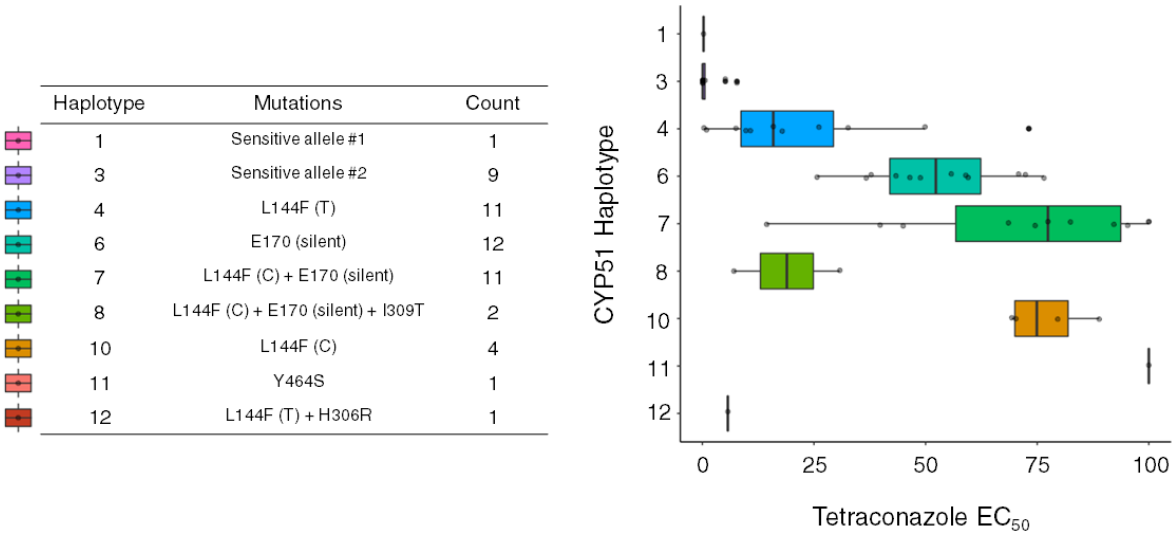


Figure 2.8. Effect of *CYP51* haplotypes on tetraconazole sensitivity in 2019 *C. beticola* strains. The left panel displays 9 different *CYP51* coding sequence haplotypes found in 52 *C. beticola* isolates from 2019. The haplotypes have consistent numbering with the 190 *C. beticola* isolates from 2016 and 2017, displayed in Figure 2.6. Mutations were identified as compared to the most common sensitive Haplotype (#3). The right panel displays box and whiskers plots for tetraconazole $EC_{50}$ values for each *CYP51* haplotype.

### 2.4.6. DMI fungicide resistance marker evaluation

In this study, we have identified multiple novel DMI fungicide resistance markers. For the future development of molecular markers for DMI fungicide resistance detection in field

isolates of *C. beticola*, we established the best minimal combination of markers to detect DMI-resistant isolates based on our collection of 190 isolates. Markers for consideration included the most common *CYP51* mutations E170 (9_1451478) and L144F (9_1451556) as well as the five significantly associated GWAS markers: 1_1890198 (RGS protein), 1_2637787 (hypothetical protein), 9_1503309 (ABC PDR transporter), 9_1452111 (upstream of *CYP51*) and 4_3725552 (DYRK protein kinase). We used the tetraconazole $EC_{50}$ threshold of 1 µg/mL or above to define an isolate as DMI-resistant, as determined experimentally by Bolton et al. (2012b). The markers 1_1890198 (RGS protein) and 4_3725552 (DYRK protein kinase) were discounted for practical use, since 56.8% and 57.9% of DMI-sensitive isolates in our study harbored the respective "resistant" alleles. The most effective marker was 1_2637787 (hypothetical protein) with the A allele present in 78.9% (75/95) of resistant isolates and just 12.6% (12/95) of sensitive isolates. Of the remaining 20 resistant isolates, the best marker was 9_1503309 (ABC PDR transporter) for which the C allele was in 55% (11/20) of isolates (70.5% of total resistant isolates and 18.9% of sensitive isolates). Of the nine remaining resistant isolates, three had 9_1451556 (L144F (T)) and one had 9_1452111 (upstream of *CYP51*, A allele). Just five of the 95 resistant isolates (5.3%) did not have the resistant allele for the four markers and did not harbor any *CYP51* mutations. Together, the four markers could identify 94.7% of the resistant isolates ($EC_{50}$ > 1µg/mL).

**2.4.7. Assessing fitness penalties for DMI fungicide resistance loci**

To investigate whether there was a fitness penalty associated with DMI fungicide resistance, we measured the radial growth rates of all 190 *C. beticola* strains as a proxy for fitness (Table 2.1). We performed association analyses for radial growth rate and obtained allelic effect estimates for each marker. There was a very weak positive correlation between allelic effects of DMI fungicide resistance (tetraconazole $EC_{50}$ values) and radial growth rate (Pearson

correlation coefficient = 0.1739), indicating that there is no obvious fitness penalty with regards to growth associated with DMI fungicide resistance (Fig. 2.9). The most significant markers associated with tetraconazole resistance also appeared to have a negligible effect on growth rate (Table 2.6). We also performed association analyses for radial growth of isolates under salt stress (1M NaCl). There was a slightly stronger positive correlation between the allelic effects of DMI fungicide resistance (tetraconazole $EC_{50}$ values) and radial growth rate under salt stress (Pearson correlation coefficient = 0.3218) (Fig. 5B). This suggests that some mechanisms of DMI fungicide resistance may also lead to increased salt tolerance. However, the most significant markers associated with tetraconazole resistance did not appear to have meaningful impact on growth rates under salt stress (Table S6, Fig. 5B).

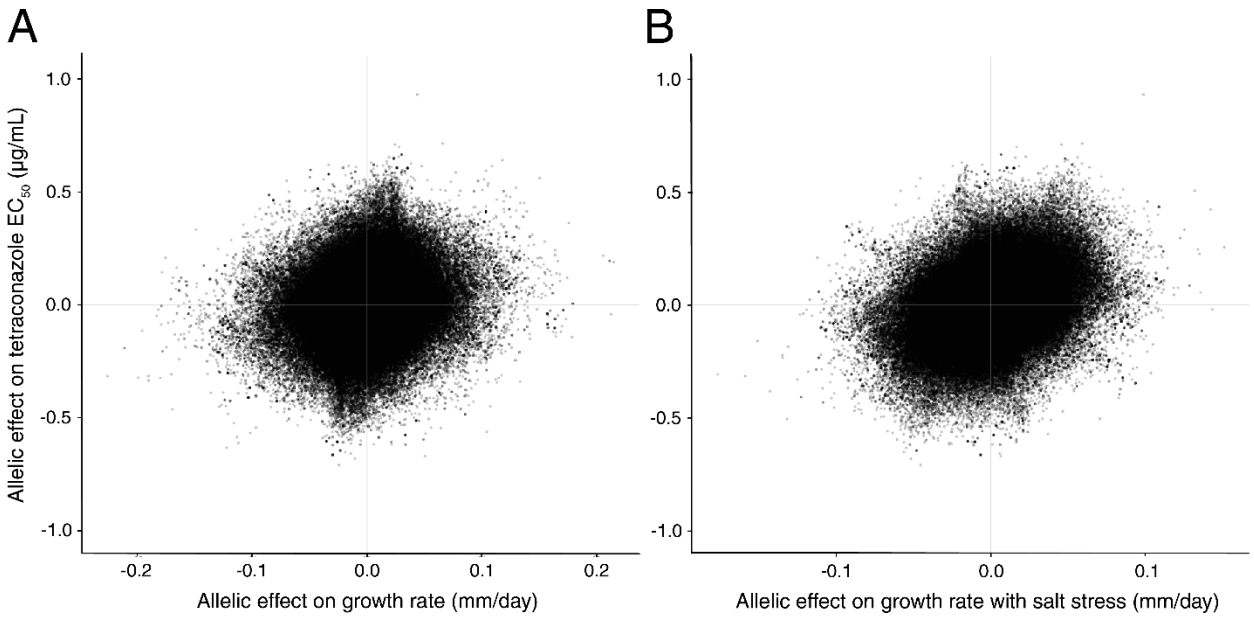


Figure 2.9. Correlation of genome-wide allelic effects on tetraconazole sensitivity ($EC_{50}$) and radial growth rates (mm/day) of *C. beticola* cultures under different conditions. The allelic effect estimates were obtained for 290,496/320,530 markers from association mapping performed in GAPIT. Pearson correlation test revealed a slight positive correlation between the allelic effects for tetraconazole sensitivity and radial growth rate (A) (coefficient = 0.1739). There was a stronger positive correlation between the allelic effects for tetraconazole sensitivity and radial growth under 1M NaCl salt stress (B) (coefficient = 0.3218).

Table 2.6. Allelic effect estimates for the most significantly associated markers with tetraconazole sensitivity.

| Chromosome | Position (bp) | Associated gene annotation | Marker type[1] | Allelic effect on tetraconazole sensitivity ($EC_{50}$)[2] | Allelic effect on growth rate (mm/day)[2] | Allelic effect on growth rate under salt stress (mm/day)[2] |
|---|---|---|---|---|---|---|
| 1 | 1890198 | Sorting nexin-12 | Indel | -0.372 | 0.003 | -0.020 |
| 1 | 2637787 | Hypothetical protein | SNP | -0.615 | -0.014 | -0.023 |
| 9 | 1503309 | ABC transporter CDR4 | SNP | -0.541 | -0.030 | -0.006 |
| 9 | 1452111 | Upstream of *CYP51* | SNP | -0.591 | -0.039 | -0.034 |
| 9 | 1503297 | ABC transporter CDR4 | SNP | -0.540 | -0.030 | -0.007 |
| 9 | 1503321 | ABC transporter CDR4 | SNP | -0.540 | -0.030 | -0.008 |
| 9 | 1503303 | ABC transporter CDR4 | SNP | 0.540 | -0.030 | 0.009 |
| 9 | 1503315 | ABC transporter CDR4 | SNP | 0.540 | -0.030 | 0.007 |
| 9 | 1503312 | ABC transporter CDR4 | SNP | -0.540 | -0.030 | -0.007 |
| 9 | 1502965 | ABC transporter CDR4 | SNP | 0.525 | 0.029 | -0.003 |
| 4 | 3725552 | Dual specificity protein kinase Pom1 | Indel | -0.376 | -0.014 | 0.005 |
| 9 | 1502968 | ABC transporter CDR4 | SNP | 0.522 | 0.027 | -0.003 |
| 9 | 1502966 | ABC transporter CDR4 | SNP | -0.522 | 0.027 | 0.003 |

[1]Type of marker used in association analyses – an indel or single nucleotide polymorphism.
[2]Allelic effect estimates on phenotype derived from association analyses in GAPIT software.

### 2.4.8. Discovery of a *CYP51-like* gene that does not appear to affect DMI sensitivity

The existence of multiple CYP51 paralogs has been described in several ascomycete fungi (Becher and Wirsel 2012; Fan et al. 2013; Hawkins et al. 2014; Liu et al. 2011). In the 09-40 *C. beticola* reference genome, there is a second gene annotated as Eburicol 14a-demethylase on chromosome 4 (NCBI XP_023452197.1) with 51.5% protein identity to CYP51. A protein homology of 40% is the minimum requirement for belonging to the same cytochrome P450 family (Nelson et al. 1993). Previous Southern blot analyses of *CYP51* suggested that there was a second P450 gene in *C. beticola* with some sequence similarity to *CYP51* (Bolton et al. 2012b). This *CYP51-like* gene is 1,530 bp and intron-free with a top NCBI BLASTp hit of a hypothetical protein in *Fonsecaea monophora* (NCBI XP_022511803.1) at 67.25% protein identity followed by multiple CYP51-like hits from *Fonsecaea*, *Cladophialophora*, *Arthrobotrys* and *Fusarium* spp. at 55-68% protein identity. Phylogenetic analyses of the *C. beticola* CYP51-like protein alongside well-characterized CYP51 proteins from other ascomycete phytopathogenic fungi placed it in a clade with *Fusarium gramineaum* CYP51C (Fig. 2.10).

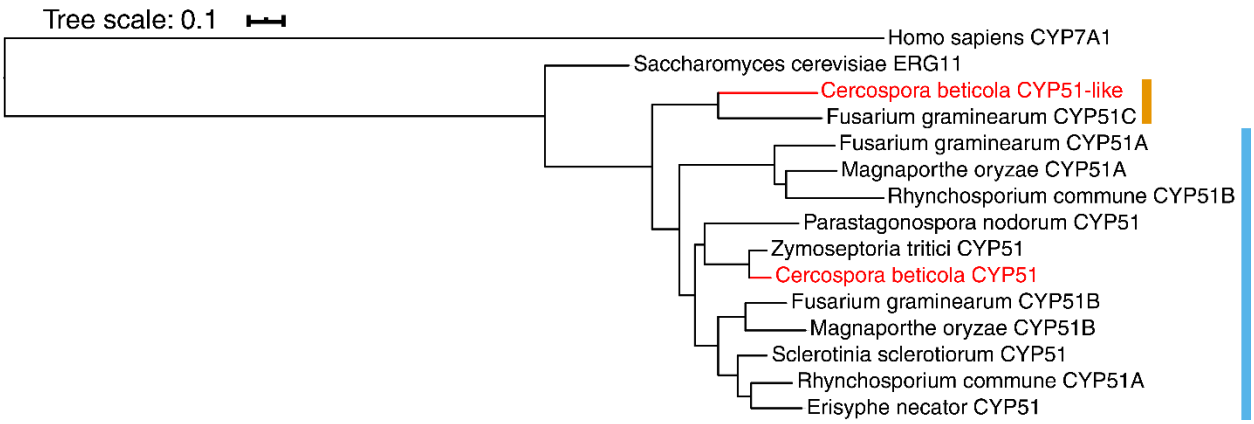


Figure 2.10. Phylogenetic analysis of *C. beticola* CYP51-like protein. Maximum-likelihood phylogenetic analysis was performed for *C. beticola* CYP51-like protein sequence alongside CbCYP51 and known CYP51 paralogs from closely related ascomycete species. The protein sequences for *Saccharomyces cerevisiae* ERG11 and *Homo sapiens* CYP7A1 were included as out-groups. CYP51-like is highlighted with a red line, where it forms a clade with *Fusarium graminearum* CYP51C (clade marked in orange). CYP51 is also highlighted with a red line, where it forms a clade with the remaining ascomycete CYP51A and CYP51B sequences (clade marked in blue).

Analysis of *CYP51-like* gene haplotypes in 190 *C. beticola* isolates revealed that seven isolates had a deletion of 1,751 bp spanning ~750 bp upstream of the ATG and the first 999 bp of coding sequence. This could be regarded as gene absence, but the remaining coding sequence could hypothetically still encode a truncated protein of 222 amino acids since there is a methionine still in frame. Overall, the deletion suggests that *CYP51-like* is non-essential or is functionally compensated by a yet-unidentified gene when absent. Several amino acid substitutions were also identified but no association could be found between *CYP51-like* gene haplotype and $EC_{50}$ value (Figure 2.11).

| Haplotype | Mutations | Count |
|---|---|---|
| 1 | 999bp deletion | 7 |
| 2 | Reference 09-40 | 120 |
| 3 | G57A | 50 |
| 4 | G57A + L224 | 5 |
| 5 | G57A + 7 more SNPs | 8 |

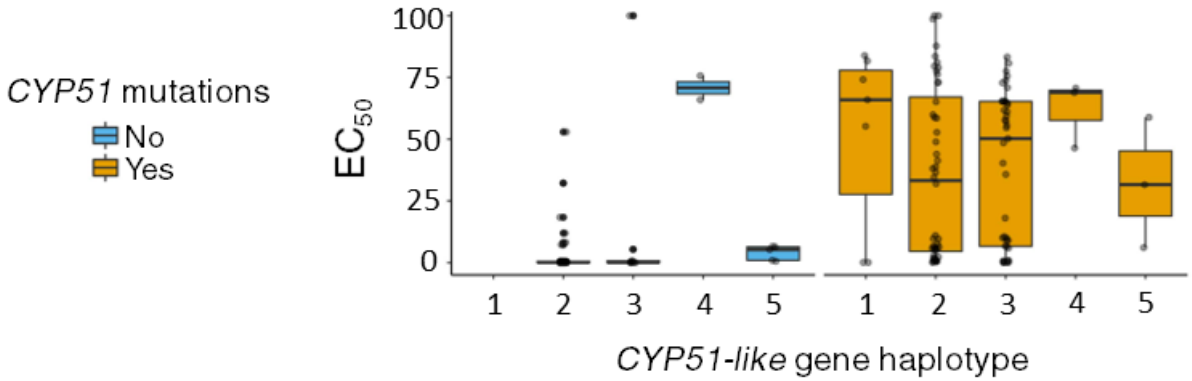


Figure 2.11. Effects of *CYP51-like* haplotypes on tetraconazole sensitivity. The table shows the five different *CYP51* coding sequence haplotypes found in our *C. beticola* population with the respective number of isolates with each haplotype. Below are box and whiskers plots for tetraconazole $EC_{50}$ values for each *CYP51-like* haplotype, grouped by the presence or absence of *CYP51* mutations.

### 2.4.9. Development of Cas9 RNP-mediated gene editing to characterize fungicide resistance mutations in *Cercospora beticola*

Current functional studies of fungicide resistance mutations within essential genes largely rely on heterologous expression of the mutant allele in yeast (Cools et al. 2010; Obuya et al. 2015). However, we hypothesized that studying the effect of synonymous mutations, such as CYP51 E170, would not be suitable in alternative hosts that ostensibly harbor differing codon biases. A split marker gene replacement method has been utilized for *C. beticola* (de Jonge et al. 2018; Ebert et al. 2019), but is not ideally suited for generating single nucleotide changes in target genes. In order to test the involvement of candidate fungicide resistance mutations, we

decided to use a gene editing method in *C. beticola*. Cas9 ribonucleoprotein-mediated gene editing was first developed by Foster et al. (2018) in *Magnaporthe oryzae*, which was adapted here using polyethylene glycol (PEG) transformation of *C. beticola* protoplasts. Briefly, Cas9 was complexed with an *in vitro*-synthesized single guide RNA (Cas9 ribonucleoprotein) and simultaneously introduced with an ~80bp donor DNA template (with the intended bp change in the center) into protoplasts via PEG transformation (Table 2.7).

Table 2.7. Sequences of sgRNAs and donor template oligonucleotides used in Cas9-RNP editing.

| Mutation | Attempt | SgRNA oligonucleotides[1] | Donor template[2] |
|---|---|---|---|
| E198A | 1 | TTCTAATACGACTCACTATAGACCTTCTGTATCGACAACGGTTTTAGAGCTAGA | GCCACTCTGTCCGTTCACCAGCTCGTCGAGAACTCCG ACGCGACCTTCTGTATCGACAACGAGGCGCTGTACG ACATTTGC |
| | 2 | TTCTAATACGACTCACTATAGTCGATACAGAAGGTCTCGTGTTTTAGAGCTAGA | |
| E170 | 1 | TTCTAATACGACTCACTATAGAAGAATTGGCGCGTTTCTTGTTTTAGAGCTAGA | CTGCTCTGCAGTCCTATGTCACATTGATCACCGAAGA AACGCGCCAATTCTTCTCTAAGAACAACCCACATAA GCGATTC |
| | 2 | TTCTAATACGACTCACTATAGGTTGTTCTTAGAGAAGAATGTTTTAGAGCTAGA | |
| | 3 | TTCTAATACGACTCACTATAGTCGAAGCGAATCGCTTATGGTTTTAGAGCTAGA | |
| L144F (C) | 1 | TTCTAATACGACTCACTATAGCTCCATGAATTTGGAGTTGGTTTTAGAGCTAGA | TTGGCAAGGACGTCGTCTACGACTGCCCCAACTCCA AATTCATGGAGCAGAAGAAGTTTGTCAAGTTCGGCT TGACCAGCG |
| | 2 | TTCTAATACGACTCACTATAGTGGGGCAGTCGTAGACGACGGTTTTAGAGCTAGA | |
| | 3 | TTCTAATACGACTCACTATAGCTGCCCCAACTCCAAATTGAGTTTTAGAGCTAGA | |
| L144F (T) | 1 | TTCTAATACGACTCACTATAGCTCCATGAATTTGGAGTTGGTTTTAGAGCTAGA | TTGGCAAGGACGTCGTCTACGACTGCCCCAACTCCA AATTTATGGAGCAGAAGAAGTTTGTCAAGTTCGGCT TGACCAGCG |
| | 2 | TTCTAATACGACTCACTATAGTGGGGCAGTCGTAGACGACGGTTTTAGAGCTAGA | |
| | 3 | TTCTAATACGACTCACTATAGCTGCCCCAACTCCAAATTGAGTTTTAGAGCTAGA | |
| Hygromycin resistance cassette insertion (intergenic) | 1 | TTCTAATACGACTCACTATAGTAAGCTTGTTAGTGTAATCAGTTTTAGAGCTAGA | PCR of pDAN with primers F: GTCGCGGGTTAAGCTTGTTAGTGTAATCAGGGTGCA ACCCGACGTTGTAAAACGACGGCCAGTG and R: GGCACGCCAACGTTAGCATTGTTAAGCTCGAGTATA GCGCCACAGGAAACAGCTATGACCATGA |
| | 2 | TTCTAATACGACTCACTATAGACGGACTGCTCCTATCGCGGTTTTAGAGCTAGA | |

[1]Oligonucleotides used to synthesize single guide RNAs *in vitro*.
[2]Donor template is double-stranded and is produced by either complexing forward and reverse complement oligonucleotides (at room temperature) or amplifying a PCR product from template DNA (hygromycin insertion cassette).

Benzimidazole fungicides target and inhibit beta-tubulin in fungi, and the E198A target site mutation is known to confer high levels of resistance in *C. beticola* (Davidson et al. 2006; Rosenzweig et al. 2015; Shrestha et al. 2020; Trkulja et al. 2013). Therefore, we initially introduced the E198A beta-tubulin mutation into a benzimidazole-sensitive strain 16-1124 to establish a working gene editing method, which resulted in a benzimidazole-resistant phenotype (Figure 2.12). Having established Cas9 ribonucleoprotein-mediated gene editing in *C. beticola*, we then attempted to introduce the L144F (TTG to TTC or TTT) and E170 (GAG to GAA) mutations into DMI-sensitive strain 16-100, using at least three different single guide RNA designs per mutation. However, all attempts to recover edited mutants failed, with none of the protoplasts growing through the tetraconazole selective media harboring the intended *CYP51* mutations. We also attempted to create these mutations using a co-editing approach, where a hygromycin resistance cassette was simultaneously introduced at a different location, allowing for simple selection on hygromycin and screening for edits at the *CYP51* locus. This method also failed to recover edited individuals. Taken together, these data suggest that we either did not identify the ideal guide RNA or donor template combination to generate *CYP51* mutations, or that the *in vivo* editing of *CYP51* in this fungus is lethal.

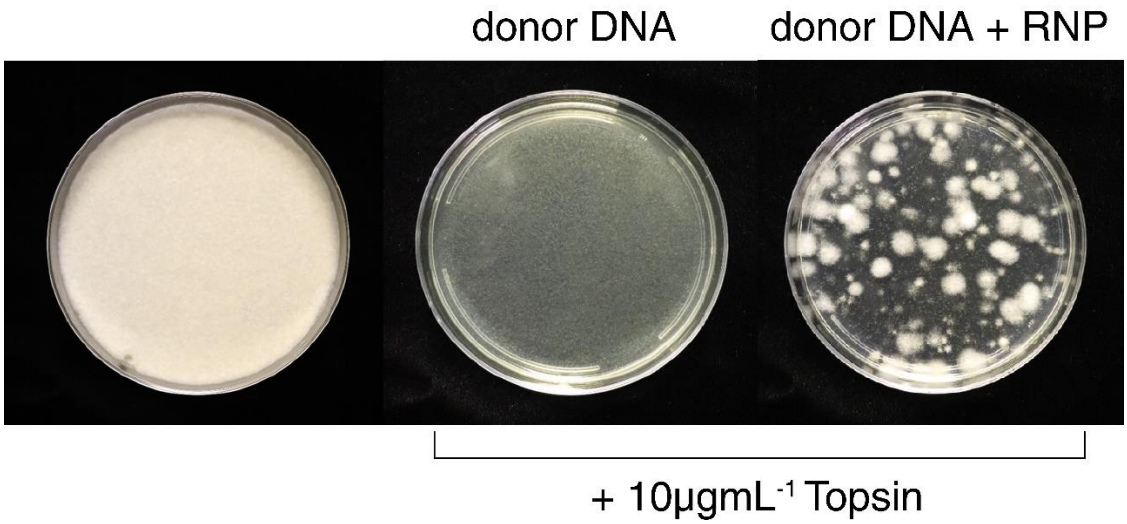


Figure 2.12. Successful use of Cas9-RNP editing in *C. beticola* to introduce the E198A mutation in beta-tubulin conferring resistance to benzimidazole fungicides. From left to right: protoplasted isolate 16-1124 grown on regeneration media without topsin, as a positive control; protoplasted isolate 16-1124 transformed with just the donor DNA and grown on regeneration media with 10µg/mL topsin, as a negative control; protoplasted isolate 16-1124 transformed with Cas9-RNP and donor DNA to introduce E198A mutation into beta-tubulin with regeneration on media containing 10µg/mL topsin.

## 2.5. Discussion

GWAS is a powerful method for identifying the genetic basis of complex phenotypic traits using variation existing within a population. Although it has been predominantly exploited for finding markers associated with disease in humans, the advent of cost-effective high-throughput sequencing technology is enabling the use of GWAS for a wide range of organisms (Power et al. 2017). Fungi are particularly amenable to GWAS because, like other microbes, they have relatively small genomes and high rates of recombination, which can increase the statistical power for complex traits (Falush 2016). For fungal crop pathogens, GWAS may have direct applications in managing disease through the discovery of key genes underlying virulence and fungicide resistance. Populations of filamentous fungi have previously been used in genome-wide association analyses to find genetic determinants of adaptive traits (Atwell et al. 2018; Ganeshan et al. 2018; Gao et al. 2016; Hartmann et al. 2017; Martin et al. 2020; Palma-

Guerrero et al. 2013; Richards et al. 2019; Zhong et al. 2017), including several studies for DMI resistance (Mohd-Assaad et al. 2016; Pereira et al. 2020; Talas et al. 2016).

In this study, we used a population of *C. beticola* strains harvested largely from the Red River Valley region of North Dakota and Minnesota, which represents the largest sugar beet production region in the U.S. DMI fungicides have been continually used since 1999 to manage *C. beticola* (Secor et al. 2010) and whilst it was initially thought that fitness penalties would preclude the spread of DMI resistance (Brown et al. 1986), resistance has increased over time leading to the occurrence of isolates with wide-ranging sensitivities and reduced disease control (Bolton et al. 2012a; Rangel et al. 2020; Secor et al. 2010). We inferred from principal component analyses that there was minor underlying population structure due to a cluster of tetraconazole-sensitive strains with more similar genetic backgrounds. Meanwhile, tetraconazole-resistant strains were generally more distantly related (Fig. 2.2B). This could be attributed to strong selection pressure exerted on North American *C. beticola* populations due to widespread and repeated use of DMI fungicides, enabling the survival and proliferation of DMI-resistant isolates, indiscriminate of genetic background. The underlying population structure explained by tetraconazole sensitivity could be confounding in downstream association mapping analyses, leading to false positive associations, and therefore underscoring why it is important to correct for this population structure in models. This observed population structure was corrected for using a kinship matrix within a mixed linear model when performing GWAS (Power et al. 2017).

GWAS identified thirteen markers on chromosomes one, four and nine associated with tetraconazole sensitivity (Fig. 2.3). These markers were associated with five distinct genes, four of which represent newly described DMI fungicide resistance mechanisms for *C. beticola* (Fig.

2.13). The most significant association was an insertion within the 5'UTR of a regulator of G-protein signalling (RGS) protein CBET3_00689 on chromosome one. The insertion appears to reduce tetraconazole sensitivity (Fig. 2.5A) and may act through up-regulation of the RGS protein. Similar RGS proteins with phosphoinositide binding Phox homology PXA and PX domains have been identified and characterized in multiple filamentous fungi (Kim et al. 2017; Wang et al. 2013; Zhang et al. 2011). Due to their intracellular signalling roles, they may be required for multiple important processes such as sexual and asexual development, stress responses, secondary metabolism and virulence (Kim et al. 2017; Zhang et al. 2011). RgsC in *Aspergillus fumigatus* was shown to be required for regular growth, asexual development, oxidative stress response, cell wall stress response, virulence and external nutrient sensing (Kim et al. 2017). MoRgs4 in *Magnaporthe oryzae* was also found to have roles in regulation of mating, conidiation, appressorium formation and pathogenicity (Zhang et al. 2011). In the case of *C. beticola,* it is possible that altered expression of the RGS domain protein may affect DMI sensitivity pleiotropically through modulation of multiple pathways such as growth, stress responses and/or metabolism such as ergosterol biosynthesis.

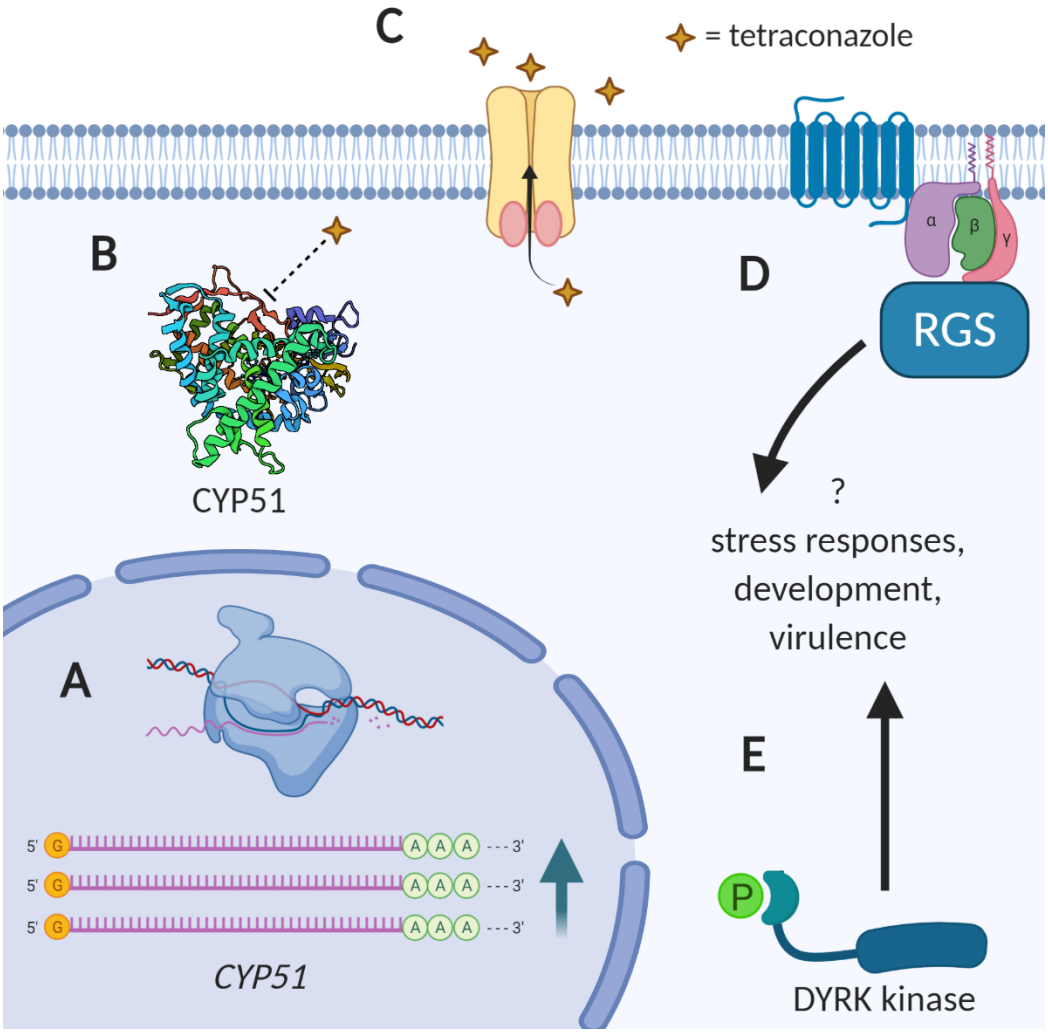


Figure 2.13. Putative mechanisms of DMI fungicide resistance in *C. beticola* (created with BioRender.com). *CYP51* induced overexpression gives rise to resistance (A) (Nikou *et al.* 2009, Bolton *et al.* 2012, 2016), as well as (B) amino acid substitutions in CYP51, leading to weakened binding and inhibition by DMIs (Trkulja *et al.* 2017, Shrestha *et al.* 2020). Multidrug transporters such as the ABC PDR transporter identified in this study may be pumping DMIs out across the membrane in a non-specific manner (C). There may be overexpression of ABC/MFS transporters such as seen in RNAseq study by Bolton *et al.* 2016, or amino acid substitutions to improve pumping of DMIs. Cellular signalling may also play a role in DMI resistance (D, E). Differential expression of a regulator of G-protein signalling (RGS) domain protein (D) or increased expression of a dual specificity DYRK protein kinase (E) may increase DMI resistance, through signalling pathways that modify expression of downstream genes such as those involved in growth, development, stress responses and/or ergosterol biosynthesis (such as *CYP51*).

A second gene on chromosome one was significantly associated with tetraconazole sensitivity. The presence of an amino acid substitution (G474S) in hypothetical protein

CB0940_00999 significantly increases tetraconazole $EC_{50}$ value (Fig. 2.3, Fig. 2.5B), even when it co-occurs with CYP51 amino acid substitutions. Since the protein lacks conserved domains, it is difficult to hypothesize its function in DMI resistance.

On chromosome nine, nine SNPs within ABC pleiotropic drug resistance (PDR) transporter gene CBET3_11399 were significantly associated with tetraconazole sensitivity. Whilst these were all synonymous or intron mutations, there are multiple other intragenic mutations nearby in complete LD that give rise to amino acid substitutions (e.g. E29D, R33Q, H47R, D152E, I210V, N218S). Most of the mutations were found within the first ~250 of 1,493 amino acids in the protein and may alter transporter function, leading to differential pumping of tetraconazole out of fungal cells (Holmes *et al.* 2002). ABC transporters, namely those with multidrug resistance (MDR) or pleiotropic drug resistance (PDR) domains, have been implicated previously in non-specific efflux of DMI fungicides in pathogenic fungi (Hamamoto et al. 2001; Hayashi et al. 2002a). Whilst overexpression of ABC transporters is a common mechanism of drug resistance in fungi (Cannon et al. 2009; Hamamoto et al. 2001; Hellin et al. 2018; Kretschmer et al. 2009; Nakaune et al. 1998; Slaven et al. 2002), there has been evidence for the involvement of point mutations affecting substrate binding and function too (Holmes et al. 2006).

The other significant locus on chromosome nine was a SNP 124bp upstream of the DMI target *CYP51*. It is possible that a single base pair change upstream of the gene could affect binding of a transcriptional regulator and in turn affect gene expression (Buckland 2006; Chen et al. 2016). *CYP51* overexpression was previously associated with DMI resistance in *C. beticola* (Bolton et al. 2012b; Bolton et al. 2016; Nikou et al. 2009). When we investigated LD in the region, we found that the silent mutation E170 in *CYP51* was in high LD with the upstream SNP

9_1452111. However, we could not uncouple the effects of these mutations on tetraconazole $EC_{50}$ value since the upstream SNP was always found with either E170 or a non-synonymous mutation in *CYP51*. We investigated the possibility of the upstream SNP influencing transcription factor binding sites using likely DNA-binding sites derived from *Saccharomyces cerevisiae* (JASPAR database). Predicted binding sites differed for both versions of the upstream SNP, suggesting that differential *CYP51* regulation could occur. Differential binding site likelihoods were found for six different transcription factors including UPC2, ECM22 and STB5 which have all been described in other fungi as transcriptional regulators of *CYP51* and ergosterol biosynthesis (Flowers et al. 2012; Silver et al. 2004; Vik and Rine 2001), or pleiotropic drug resistance (Noble et al. 2013). It is possible that homologous transcriptional regulators in *C. beticola* differentially bind to the *CYP51* promoter due to the SNP (9_1452111), leading to up-regulation and reduced DMI sensitivity.

The fifth most significantly associated locus was an indel in the 5'UTR of dual-specificity DYRK protein kinase CBET3_05141 on chromosome 4. The insertion appears to contribute to increased tetraconazole $EC_{50}$ value. Intriguingly, RNAseq performed previously (Bolton et al. 2016) revealed that this gene was differentially up-regulated in a tetraconazole-resistant strain in response to tetraconazole exposure, when compared to a more sensitive strain. Together, this suggests that the 5'UTR insertion causes up-regulation of the protein kinase gene in response to tetraconazole. DYRK protein kinases are an evolutionarily conserved family of protein kinases which regulate cell growth and differentiation, such as Pom1 in fission yeast (Becker et al. 1998). DYRK family members phosphorylate many different substrates, including critical regulators of the cell cycle, and downstream effects include increased transcription factor activity, modulation of subcellular protein distribution and regulation of enzyme activity (Becker

2012). The increased expression of this DYRK protein kinase in *C. beticola* could lead to reduced DMI sensitivity through multiple pleiotropic effects downstream of its signalling pathway.

We also demonstrated that the 13 tetraconazole sensitivity markers identified through GWAS had little effect on radial growth rate (Table 2.4). Considering fungal growth rate as a proxy for fitness, this suggests that there is no obvious fitness penalty for these DMI fungicide resistance loci. This was corroborated by finding no clear correlation between genome-wide allelic effects on tetraconazole $EC_{50}$ values and radial growth rates (Fig. 2.6). Nikou et al. (2009) found no differences in radial growth, sporulation, or pathogenicity on sugar beet for five European *C. beticola* isolates resistant to DMI fungicide epoxiconazole (3 µg/mL < $EC_{50}$ < 6 µg/mL), when compared to a sensitive isolate ($EC_{50}$=0.05 µg/mL). In another study by (Bolton et al. 2012a), US *C. beticola* isolates grouped by low, medium or high tetraconazole $EC_{50}$ values showed no differences in CLS disease severity on sugar beet. However, an older study by Karaoglanidis et al. (2001) showed that European *C. beticola* isolates resistant to flutriafol (2.7 µg/mL $\leq EC_{50} \leq$ 15.6 µg/mL) had significantly lower spore production and virulence on sugar beet than sensitive isolates (0.10 µg/mL $\leq EC_{50} \leq$ 0.34 µg/mL). Competition assays performed on sugar beet in the field between sensitive and resistant isolates also resulted in a significant reduction in frequency of resistant isolates (Karaoglanidis et al. 2001). It is possible that a trade-off exists for DMI fungicide resistance in *C. beticola*, but we have not observed it with the proxy phenotype and conditions tested in this study.

Genome-wide allelic effects on radial growth rate under salt stress (1M NaCl) had a slight positive correlation with allelic effects on tetraconazole $EC_{50}$ (Fig. 5B). This suggested that some mechanisms conferring increased DMI fungicide resistance may also lead to enhanced salt

tolerance. Fungal strategies for overcoming salt stress include morphological changes, cell wall reinforcement and accumulation of osmolytes such as glycerol (Liu et al. 2017). In some fungal species, there are common signaling components controlling both salt stress responses and antifungal drug resistance (Hayes et al. 2014), including the high osmolarity glycerol (HOG) response pathway (Kim et al. 2011; Zhang et al. 2002) and calcineurin signaling pathway (Jacob et al. 2015; Juvvadi et al. 2014). Genetic variants leading to enhanced signaling in response to stress may lead to both increased salt and fungicide tolerance in *C. beticola*.

With the possibility of *CYP51* mutations being associated with DMI resistance, we examined the gene sequence within all 190 GWAS isolates, and an extra 52 2019 *C. beticola* isolates. In this study, there were two main haplotypes associated with low tetraconazole $EC_{50}$ values: a common haplotype found in 85 isolates, and a highly divergent sequence with 114 polymorphisms shared by just three isolates. Since *C. apii* is morphologically indistinguishable from *C. beticola* (Groenewald et al. 2005), we questioned whether these three isolates might be another species. However, whole genome phylogenetic analyses, as well as analysis of calmodulin, TEF and actin genes identified these isolates as *C. beticola* (not shown). Compared to the most common sensitive haplotype, there were two synonymous mutations (I122 and E170) and six different non-synonymous mutations: L144F, H306R, I309T, I387M, Y464S and V467A. The L144F, I387M and Y464S mutations were associated with increased tetraconazole $EC_{50}$ value and so may be directly involved in DMI resistance.

In fungal human and plant pathogens, evolutionary flexibility of CYP51 to accept structural changes has often led to the accumulation of amino acid changes and selection of haplotypes that reduce DMI binding and inhibition (Becher and Wirsel 2012). The amino acid substitutions L144F, I387M and Y464S were previously reported in *C. beticola* strains from

Serbia and, as in our study, were individually associated with DMI resistance (Trkulja et al. 2017). Mair et al. (2016) proposed a system for unifying labelling of amino acids in CYP51 through alignments between CYP51 proteins in relevant species fitted to the well-studied *Z. tritici* 'archetype'. Orthologous amino acids in all species can then be consistently labelled based on the position of the amino acid in the archetype protein. We found that L144F was the most common CYP51 amino acid change in RRV *C. beticola* isolates from 2017, 2018 (Fig. 2.7) and 2019 (Fig. 2.8), which has not been reported in orthologous sites in other fungal species (Mair et al. 2016). The I387M mutation (haplotype 9) also does not appear to have orthologous mutations in other fungi. However, the Y464S mutation appears to be analogous to Y461S/G/H in *Z. tritici* (Cools and Fraaije 2012; Mair et al. 2016). In *Z. tritici*, changes in residues Y459 to Y461 occur frequently (Cools and Fraaije 2012). Additionally, alterations in equivalent residues in Y459 to Y461 have been found in *A. fumigatus* (Howard et al. 2006), *C. albicans* (Perea et al. 2001) and *Mycosphaerella fijiensis* (Cañas-Gutiérrez et al. 2009), all of which were associated with increased resistance to DMIs. Expression of *ZtCYP51* encoding Y461H in *S. cerevisiae* confers decreased sensitivity to all DMIs (Cools et al. 2010). Molecular modelling predicted this residue to be integral to the CYP51 active site with alterations directly impacting DMI binding (Mullins et al. 2011). Despite the widespread association of residues Y459 to Y461 to DMI resistance in fungal species, the Y464S amino acid exchange was not common in our study with only two isolates harboring this mutation.

To our knowledge, we also present three novel CYP51 amino acid substitutions in *C. beticola*, H306R, I309T and V467A. The impact of these relatively rare mutations is still unclear. A single isolate from 2019 harbored H306R, and it was present alongside L144F which is individually associated with DMI resistance. Only two isolates had I309T and the substitution

was co-present with L144F and E170, which are both individually associated with DMI resistance. No analogous mutations for H306R or I309T have been found in other fungal species (not shown). Likewise, only one isolate in our study had the V467A substitution and it was present alongside the silent mutation I122. Intriguingly, this isolate was highly sensitive to DMIs, suggesting that V467A, although close to the Y464 residue, does not affect binding to CYP51.

Unexpectedly, we discovered a potential codon usage effect for the L144F substitution in CbCYP51. We observed that strains with L144F encoded by the TTT codon had a significantly lower $EC_{50}$ value than strains with L144F encoded by the TTC codon. We did not find another mutation within or close to *CYP51* (±1 kb) in LD with the codon difference. In *C. beticola*, the phenylalanine codon TTT is used just 30% of the time in coding sequence when compared to the codon TTC at 70%, representing the biggest difference in codon usage for a single amino acid in *C. beticola*. The model fungus *N. crassa* exhibits a similar codon bias for phenylalanine with TTC used in ~67% of cases (Kazusa codon usage database). The use of rare vs. optimal codons in *N. crassa* has been shown to impact transcript levels (Zhou et al. 2016; Zhou et al. 2018), protein abundance (Zhou et al. 2015) and co-translational folding of proteins (Yu et al. 2015). Since we did not observe differences in *CYP51* expression levels between L144F strains (data not shown), we suspect that the codon usage here instead impacts CYP51 protein folding or abundance. It has long been suggested that codon usage regulates protein folding by affecting the rate of translation (Goldman et al. 1995; Komar et al. 1999; Konigsberg and Godson 1983; Sørensen et al. 1989; Zhou et al. 2009). For example, a synonymous mutation to a rare codon within the human *multidrug resistance 1* (*MDR1*) gene results in an altered protein conformation and modified drug interactions (Kimchi-Sarfaty et al. 2007). The change in isoleucine codon

from ATC (47% usage) to rarer codon ATT (35% usage) gave differential MDR1 protein conformation and decreased the ability of an efflux blocker to inhibit MDR1. An even rarer isoleucine codon ATA (18% usage) further decreased the inhibitor effects on MDR1. The authors hypothesized that the effect was due to codon context; when frequent codons are changed to rare codons within a cluster of rare codons, the timing of co-translational protein folding is affected (Anthony and Skach 2002). Zhou et al. (2015) used the filamentous fungus *Neurospora crassa* and manipulation of codons in the circadian clock gene *frequency* (*frq*) to demonstrate a correlation between codon usage and protein disorder tendency. In this context, rarer codons are preferentially used in intrinsically disordered regions whereas more optimal codons are used in structured domains. We hypothesize that the phenylalanine codon TTT at position 144 of *CYP51* in *C. beticola* yields a different protein structure than codon TTC, which in turn causes differential binding of DMIs. Another possibility is that the optimized TTC codon leads to a higher overall translation rate of CYP51 (due to tRNA availability) and the higher protein level results in reduced DMI sensitivity. Functional studies will be necessary to confirm these hypotheses.

Intriguingly, we identified a silent mutation (E170) to be associated with DMI resistance in our study. Obuya et al. (2015) also associated this mutation with DMI resistance using RRV isolates, and it was also previously found to be associated with resistance in *C. beticola* in isolates from Greece (Nikou et al. 2009) and Serbia (Trkulja et al. 2017). Obuya et al. (2015) heterologously expressed a *C. beticola CYP51* haplotype harboring E170 in *Saccharomyces cerevisiae* strain R1 lacking multidrug resistance transporter Pdr5 (ΔPdr-5) and found no change in DMI sensitivity. However, it is possible that this mutation has a *C. beticola*-specific influence on DMI sensitivity through codon usage, and thus functional studies in alternative hosts may not

be conclusive. For glutamic acid (E), the GAG codon seen in more DMI-sensitive strains is used slightly more often (56%) than the GAA codon (44%). It is possible that codon usage in this context leads to differential co-translational CYP51 folding, protein structure and DMI binding as suggested above for L144F. Since the GAA codon found in resistant strains is the nonoptimal codon, it seems unlikely that it would increase the translation rate and CYP51 protein levels. Another possibility is that the synonymous change influences DMI resistance via *CYP51* expression levels e.g. via promotion of premature transcription termination (Zhou et al. 2018), chromatin structure (Zhou et al. 2016), mRNA stability (Duan and Antezana 2003) or even small RNA-based gene regulation (Lee et al. 2010). Interestingly, a synonymous mutation in *CYP51* from the grapevine powdery mildew pathogen *E. necator* was only identified in isolates with high *CYP51* expression and increased resistance to DMIs (Rallos and Baudoin 2016). In humans, over 50 diseases are associated with synonymous mutations (Sauna and Kimchi-Sarfaty 2011) which have been linked to aberrant splicing, mRNA stability or codon bias effects. There are also at least five synonymous mutations in *Escherichia coli* protein TEM-1 β-lactamase that confer higher total and functional protein levels and increase resistance to cefotaxamine (Zwart et al. 2018).

Nikou et al. (2009) found that four highly DMI-resistant isolates harbored E170 and also overexpressed *CYP51*. In the present study, we observed that strains with E170 tended to have higher *CYP51* expression, but the mean value was not statistically significant from that of sensitive strains. Alternatively, the E170 mutation may be in LD with the upstream SNP 9_1452111 which itself could affect *CYP51* gene expression and be directly involved in DMI resistance. However, since isolates from disparate locations including the RRV (Obuya et al. 2015), Greece (Nikou et al. 2009), and Serbia (Trkulja et al. 2017) have identified an association

between E170 and DMI resistance, it is tempting to speculate a direct involvement between this mutation and DMI resistance. Functional studies will be necessary to confirm the involvement of E170 with DMI resistance.

In some ascomycete fungi, there are two *CYP51* paralogs known as *CYP51A* and *CYP51B* that both have sterol 14 α-demethylase function in ergosterol biosynthesis (Brunner et al. 2016). This is the case in multiple plant pathogenic fungi such as *R. commune* (Hawkins et al. 2014), *Pyrenophora teres* f. sp. *teres* (Mair et al. 2016), *F. graminearum* (Liu et al. 2011), *Penicillium digitatum* (Sun et al. 2011) and *Colletotrichum* spp. (Chen et al. 2020). DMI use can impose selection on favorable mutations within or upstream of either paralog. We identified a possible CYP51 paralog, CYP51-like, in the 09-40 *C. beticola* reference genome which had 51.4% protein similarity to CYP51. Analysis of *CYP51-like* haplotypes in our population suggests that the gene is not associated with sensitivity to tetraconazole or other DMI fungicides tested. Seven isolates had a deletion in *CYP51-like* giving partial gene absence, suggesting that the gene is non-essential or that functional redundancy exists. Phylogenetic and BLASTp analyses of protein sequences demonstrated that CYP51-like is more similar to CYP51C found in *Fusarium* spp. than CYP51 from *C. beticola* or paralogs from more closely related ascomycetes. The *CYP51C* gene was previously considered as being unique to *Fusarium* spp. (Fernández-Ortuño et al. 2010). Fan et al. (2013) demonstrated that CYP51C in *F. graminearum* had functionally diversified from the other CYP51A and CYP51B, since it failed to complement yeast CYP51 function and it was required for full virulence on wheat ears. It is possible that CYP51-like in *C. beticola* also lacks sterol 14α-demethylase function and plays another role in the fungus, which could be elucidated through the generation of deletion mutants.

In addition to determining multiple genomic loci associated with DMI fungicide resistance, we identified a set of four markers from our study that could be used in combination to identify ~95% of the resistant isolates in our study: 1_2637787 (hypothetical protein), 9_1503309 (ABC transporter), 9_1451556 (L144F) and 9_1452111 (upstream of *CYP51*). An important outcome of this work is to develop molecular markers for quick and accurate detection of DMI-resistant field isolates of *C. beticola*. A probe-based qPCR assay was already developed by our lab for the L144F mutation in *CYP51*, 9_1451556 (Shrestha et al. 2020). We recommend that the remaining three markers be developed for rapid qPCR or LAMP assay, as described by Shrestha et al. (2020) and be evaluated on a much larger population to establish their reliability.

As mentioned above, GWAS is a powerful tool to identify genomic variants associated with a trait of interest. However, to establish causality the gold standard is functional validation. Gene replacement methods are useful for establishing gene function. For genes of known function, such as *CYP51*, we are often interested in the effects of specific mutations which requires other methods such as heterologous expression of *CYP51* haplotypes in yeast (Cools et al. 2010). However, it is possible that mutations only give an observed phenotypic change when expressed in the native species e.g. due to codon usage bias. Genome editing provides an elegant way of introducing desired mutations into a target gene in the native species, without the need for co-introduction of a selectable marker if the mutation itself is selectable. Therefore, this method lends itself to investigating mutations involved in fungicide resistance. The *CYP51* gene has been edited in human fungal pathogens *Aspergillus fumigatus* (Umeyama et al. 2018) and *Candida* spp. (Morio et al. 2019) which exhibit azole resistance. To our knowledge, this has not yet been done in phytopathogenic fungi. We adapted a Cas9-RNP gene editing method developed by Foster et al. (2018) for use in *C. beticola* to functionally assess fungicide resistance

mutations. To establish this method in our laboratory, we first edited the beta-tubulin gene to introduce the E198A mutation associated with benzimidazole resistance (Davidson et al. 2006; Shrestha et al. 2020).We successfully introduced the E198A mutation into a benzimidazole-sensitive *C. beticola* strain and recovered mutants using selection on benzimidazole-amended media. Despite these initial successes, we were unable to introduce L144F (TTT or TTC) or E170 mutations into CYP51. Likewise, attempts to co-edit through simultaneous introduction of a hygromycin-selectable marker at a different genomic location were also not successful. It is unclear why it was not possible for us to edit *CYP51*, but we conclude that we either did not find optimal sgRNA and template sequences or that editing the essential gene *CYP51* is lethal in *C. beticola*. An alternative strategy would be to generate a viable *ΔCYP51* knockout mutant by supplementing the media with ergosterol and transforming the mutant with different *CYP51* haplotypes to test their effects.

### 2.6. Conclusion

To conclude, GWAS was used for the first time in a *Cercospora* species. We identified mutations on chromosomes one, four and nine associated with DMI fungicide resistance within or upstream of five different genes: an RGS domain protein, a dual specificity DYRK-type protein kinase, an ABC transporter, a hypothetical protein and the DMI target, *CYP51*. There was no evidence of a fitness trade-off for DMI fungicide resistance, using radial growth as a proxy. Haplotype analysis of *CYP51* demonstrated that intragenic mutations E170, L144F, I387M and Y464S are significantly associated with tetraconazole resistance. Future studies should establish if the mutations identified are directly involved in DMI fungicide resistance and clarify the role of *CYP51* overexpression. Cas9-RNP editing, although successful for beta-tubulin, did not allow us to functionally assess CYP51 mutations in *C. beticola*. Overall, we have

demonstrated that GWAS can be useful even for local populations of *C. beticola*. The identification of markers associated with DMI resistance has allowed for the development of methodologies to identify resistant strains (Shrestha et al. 2020), which was a major goal for this study, and additional markers from this study can be developed for future use. Moreover, the available isolate genotyping data could be used in future GWAS studies to establish the genetic architecture of other traits of importance, including virulence on the sugar beet host.

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# CHAPTER 3. SEED-BORNE *CERCOSPORA BETICOLA* CAN INITIATE CERCOSPORA LEAF SPOT IN SUGAR BEET (*BETA VULGARIS* L.)[3]

## 3.1. Abstract

Cercospora leaf spot (CLS) is a globally important foliar disease of sugar beet (*Beta vulgaris* ssp. *vulgaris*) caused by the hemi-biotrophic fungus *Cercospora beticola*. Management of CLS has been challenging due to the polycyclic nature of the disease and the rapid development of resistance to many fungicides. Long-distance movement of *C. beticola* has been indirectly evidenced in multiple recent population genetic studies. In this study, we provide direct evidence for seed-borne *C. beticola* to initiate disease in sugar beet. We confirmed the presence of viable *C. beticola* for ten of 37 sugar beet seed lots in plate growth assays. Each of the 38 seed-derived *C. beticola* isolates had a unique genotype over eight microsatellite loci. All isolates contained the G143A mutation in cytochrome b conferring QoI fungicide resistance, and 32 of 38 isolates showed low DMI fungicide sensitivity ($EC_{50}$ > 1µg/mL). Direct planting of pelleted seed demonstrated the ability of seed-borne inoculum to initiate CLS disease in sugar beet. *C. beticola* DNA was detected in DNA isolated from xylem sap, suggesting that it may use the vascular system to systemically colonize the host sugar beet plant. Additionally, this was the first study to investigate the fungal microbiome within sugar beet seed. Long-read internal

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transcribed spacer amplicon sequencing using the MinION platform demonstrated the presence of potentially viable fungi from 19 different fungal genera. *Fusarium*, *Alternaria*, and *Cercospora* were dominant taxa and comprised an average of 93% relative abundance over 11 seed lots. The presence of seed-borne inoculum should be considered when implementing integrated disease management strategies for CLS of sugar beet in the future. Further investigation is required to develop reliable diagnostic tools for seed health inspection and to study the epidemiology and control of this primary inoculum source.

## 3.2. Introduction

Cercospora leaf spot (CLS) is the most destructive foliar disease of sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) worldwide and is caused by the fungus *Cercospora beticola* (Rangel et al. 2020). In warm temperate growing regions, CLS can cause up to a 50% loss in recoverable sucrose (Lamey et al. 1987) and infected plants are more susceptible to post-harvest disease in storage piles (Shane and Teng 1992). Disease management programs for CLS currently integrate cultural practices, host genetic resistance, and fungicide applications (Rangel et al. 2020). Cultural practices, such as rotation with non-host crops and tillage to bury infested plant debris, aim to reduce primary inoculum for the next season. The development of sugar beet varieties with CLS tolerance that are high yielding has historically been a challenge, but continual improvements are being made (Smith and Campbell 1996; Vogel et al. 2018). In growing regions where disease pressure is high, the current forms of genetic-based host resistance are insufficient to manage CLS alone (Rangel et al. 2020). Consequently, fungicide applications from several chemical classes are required in a timely manner to inhibit the proliferation of *C. beticola* in sugar beet leaves. However, widespread and repeated use of the same fungicides over large growing areas has rapidly led to the development of resistance to several chemical classes in *C. beticola* populations (Birla et al. 2012; Bolton et al. 2012a; Bolton et al. 2013; Rangel et

al. 2020; Secor et al. 2010). Management of fungicide resistance through mixing and rotation of different chemical classes (Fungicide Resistance Action Committee (FRAC) groups) is aiding in the retention of fungicide efficacy (Secor et al. 2010), but additional strategies are required to enhance the sustainability of this tactic.

Many questions remain regarding the epidemiology of CLS disease. The dominant primary inoculum for local epidemics was considered to be specialized fungal overwintering structures on plant debris called pseudostromata (Khan et al. 2008; McKay and Pool 1918; Pool and McKay 1916). Pseudostromata can persist on sugar beet or alternative host plant tissue (Knight et al. 2019a) for over three years if not incorporated into the soil (Khan et al. 2008). In the spring, conidia can form on pseudostromata to initiate the infection process, and multiple asexual cycles can occur within a single season (McKay and Pool 1918; Nagel 1945; Vereijssen et al. 2007). Several population genetics studies in *C. beticola* have questioned the solo role of clonally reproducing primary inoculum in CLS (Groenewald et al. 2008; Knight et al. 2018; Knight et al. 2019b; Vaghefi et al. 2017a; Vaghefi et al. 2017c). A teleomorph has not been found for *C. beticola*, but genetic evidence has supported panmictic populations (Bolton et al. 2012c; Groenewald et al. 2006; Groenewald et al. 2008; Vaghefi et al. 2017c). Recurrent clonal lineages have also been found across continents (Knight et al. 2019b; Vaghefi et al. 2017c), suggesting the long-distance movement of *C. beticola*. However, spore dispersal studies have indicated a limited range of *C. beticola* (Imbusch et al. 2019), suggesting that long-distance movement mediated by spores is not likely. Studies have also linked higher genetic differentiation in *C. beticola* populations both spatially (Vaghefi et al. 2017a) and temporally (Knight et al. 2018) to changing seed sources.

Humans have a long history of mediating the long-distance dispersal of pathogens. For example, plant-pathogenic fungi have been associated with seed lots including *Claviceps purpurea* of rye as described by Hellwig in 1699 (K F Baker and Smith 1966) and *Colletotrichum lindemuthianum* of bean as described by Frank in 1883 (Dey 1919). Multiple fungal pathogens have been associated with sugar beet seed including *Neocamarosporium betae* (syn. *Phoma betae*, *Pleospora betae*) causing Phoma leaf spot and root rot and *Uromyces betae* causing beet rust (Agarwal et al. 2006; Richardson 1990). *Cercospora beticola* has also been reported to be seed-borne in sugar beet (McKay and Pool 1918). McKay and Pool (1918) identified a seed lot, generated from sugar beet with CLS, that was infested with viable *C. beticola* conidia. When sown, the seedlings produced from this seed source had CLS lesions on the cotyledons, and formaldehyde treatment of seed reduced disease incidence. Vereijssen et al. (2004) provided anecdotal evidence of infested seed (polished, processed and pelleted) associated with CLS epidemics in Europe. Vereijssen et al. (2004; 2005) also demonstrated that sugar beet roots could act as a primary infection site for *C. beticola* conidia. Most recently, Knight et al. (2020) identified viable *C. beticola* in multiple commercial table beet seed lots. Plants grown from these infested lots developed CLS, suggesting that seed-borne *C. beticola* can cause disease in table beet.

We began to suspect that seed transmission may play a role in CLS disease of sugar beet after we identified Swedish isolates with quinone outside inhibitor (QoI) fungicide resistance in 2011, despite QoI fungicides not being used for sugar beet disease management in Sweden before 2012 (Anne Lisbet Hansen, pers.common). The QoI-resistant isolates were identified by detecting the G143A mutation in the fungal protein cytochrome b, which is associated with QoI fungicide resistance in *C. beticola* (Birla et al. 2012; Bolton et al. 2013). Long-distance

movement of these QoI-resistant isolates via infested seed from seed production areas where QoI fungicides were utilized was hypothesized as a logical explanation for these observations. Alternatively, QoI fungicide usage in non-sugar beet crops may have selected for QoI-resistant *C. beticola* strains growing saprophytically in Swedish soils or on alternative weedy hosts. In order to shed light on the potential for CLS to be propagated by seed, we set out to (i) establish whether *C. beticola* is present and viable in sugar beet seed and if so, (ii) determine if this inoculum can initiate CLS and determine if seed-derived *C. beticola* isolates are resistant to widely-used fungicide chemistries.

## 3.3. Materials and methods

### 3.3.1. Baiting and identification of fungi from sugar beet seed

Commercial sugar beet seed producers typically incorporate antifungal and/or growth-inducing chemistries in the form of a 'pellet' that surrounds the raw sugar beet seed. To initially assess whether sugar beet seed may harbor *C. beticola*, 37 different sugar beet seed lots deriving from both European and US cultivars that were commercially available were screened for fungal growth by placing un-pelleted seed on potato dextrose agar (PDA; BD Biosciences; San Jose, CA, USA) Petri plates and incubating at 22°C for 14 days. To remove pellets from seed, pelleted seeds were placed in sterile water. After 5 min, a gentle vortex was used to remove pellet from the seed. The raw seed was then surface-sterilized for 10 min by placing seed in 10% bleach (v/v) followed by triple rinsing in sterile water. Plates were monitored daily for up to 3 weeks for fungal growth. If fungal growth occurred, a single 5 mm plug was excised from the culture and used to extract DNA via a rapid sodium dodecyl sulfate (SDS) lysis prep (Fran Lopez Ruiz, Curtin University, personal communication). Growth was confirmed to be *Cercospora beticola* by performing species-specific qPCR of the calmodulin gene (CbCAL) using the primers and method described by Knight and Pethybridge (2020). Sanger sequencing of PCR products was

performed by MCLAB (San Francisco, CA, USA). The sequence obtained was assembled and a BLASTn search of the NCBI nucleotide collection was performed to confirm *C. beticola* as the top hit.

### 3.3.2. Fungicide resistance profiling of *C. beticola*

Thirty eight fungal isolates identified as *C. beticola* were single spore purified (Secor and Rivera 2012) and tested for sensitivity to selected fungicides. The G143A mutation conferring QoI fungicide resistance was assessed using the real-time PCR method described by Bolton et al. (2013). To measure sensitivity to the DMI fungicide tetraconazole, $EC_{50}$ values were measured as described by Secor and Rivera (2012). Briefly, 4 mm plugs of the isolates were placed on both unamended clarified V8 (CV8) agar plates (10% v/v clarified V8 juice (Campbell's Soup Co.), 0.5% w/v $CaCO_3$, 1.5% w/v agar (Sigma-Aldrich; St. Louis, Missouri, USA)) and CV8 plates amended with ten-fold dilutions of technical grade tetraconazole from 0.1 to 100 µg/mL. Growth rates were measured and used to calculate $EC_{50}$ values (Secor and Rivera 2012).

### 3.3.3. Genotypic diversity of *C. beticola*

To assess the genotypic diversity of the 38 *C. beticola* isolates obtained from sugar beet seed, eight polymorphic markers SSRCb20, SSRCb21, SSRCb22, SSRCb23, SSRCb24, SSRCb25, SSRCb26 and SSRCb27 (Vaghefi et al. 2017b) were amplified in multiplex PCR as described by Vaghefi et al. (2017b) using the *C. beticola* DNA detailed above. In brief, PCRs used 1X Multiplex PCR Master Mix (New England Biolabs, Ipswich, MA, USA) in a total volume of 17 µL with 0.2 µM of each primer, 1.25 U of GoTaq DNA polymerase and ~10 ng of genomic DNA. PCR conditions were an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, and with a final 7 min elongation step at 72°C. Separation of labeled DNA fragments was performed by MCLAB (South San Francisco, CA) using a Genetic Analyzer 3730*xl* (Applied Biosystems, Foster City, CA, USA). Fragment

size in base pairs was determined using Peak Scanner software (v.1.0; Applied Biosystems). Strain-specific SSR genotypes were established based on unique combinations of allele sizes obtained for the eight loci (Vaghefi et al. 2017b). Each genotype was represented by a unique two-letter combination to easily distinguish between genotypes.

### 3.3.4. Seed-to-seedling transmission efficiency assays

Twelve pelleted sugar beet seeds from seed lots 1, 3, and 10 were directly sown in 15 cm pots containing Pro-Mix BX potting soil (Quakertown, PA, USA). Pots were placed in 90 to 95% relative humidity at 30°C for approximately 13 weeks until leaves were harvested. The number of CLS lesions on each leaf was counted and averaged on a per plant basis. Harvested leaves underwent CLS lesion counts, and *C. beticola* was re-isolated from lesions as described by Secor and Rivera (2012). If lesions developed, a minimum of five lesions per seed lot underwent fungal re-isolation. *Cercospora beticola* was confirmed using the methodology described by Knight and Pethybridge (2020). The experiment was conducted twice.

### 3.3.5. Xylem sap harvest and analysis

As CLS could be initiated from sugar beet seeds, we questioned if the fungus was using xylem vessels to travel from seed to foliar tissue. To assess this, 22 pelleted sugar beet seeds from each of seed lots 1, 3, and 10 were directly sown in 15 cm diameter cones containing Pro-Mix BX potting soil (Quakertown). Cones were placed in 90 to 95% relative humidity at 30°C for five weeks at which time the hypocotyl was surface sterilized with bleach (10% v/v). Stems were then cut approximately 1 cm above the root and xylem sap was collected as it pooled on the cut surface. Xylem sap was collected for 4 h with a pipette and placed into a sterile container on ice. Xylem sap from each plant in a seed lot was pooled together to form one sample per seed lot. After the collection period, xylem sap was frozen at -20°C until further analysis. To assess whether *C. beticola* could be identified in the collected xylem sap, DNA was isolated from the

sap using an SDS lysis prep (Fran Lopez Ruiz, Curtin University, personal communication) and used in *C. beticola*-specific qPCR analyses as described by Knight and Pethybridge (2020). qPCR products were subsequently sequenced by MCLAB. The experiment was conducted twice.

### 3.3.6. MinION sequencing of seed DNA to identify seed-associated fungi

To catalog total fungal genera present in the seed, we utilized long-read sequencing of ITS PCR products using the MinION (Oxford Nanopore Technologies, Oxford, UK) sequencing platform for 11 selected sugar beet seed lots. For each seed lot, three replications were analyzed. Each replication was comprised of DNA isolated from 20 un-pelleted and surface-sterilized seed. Raw seed were ground in a mixer mill (Retsch USA; Newtown, PA, USA) using BBs to grind and homogenize the samples. DNA was isolated from ground seed using the DNeasy Plant Mini Kit (Qiagen; Germantown, MD, USA) following the manufacturer's recommendations. The full-length internal transcribed spacer regions (*ITS1* and *ITS2*) of ribosomal RNA genes were then amplified and sequenced using standard conditions with universal ITS primers ITS1f-Kyo2 and LR3-I, used by Mafune et al. (2019). The PCR included a peptide nucleic acid (PNA) blocking primer 5'-CTTTGGGTTGTGCCAGC-3' that we designed to inhibit amplification of sugar beet sequence. We obtained the sugar beet DNA sequence between ITS primers ITS1f-Kyo2 and LR3-I and used a similar approach to Lundberg *et al.* (2013) to design an elongation arrest PNA primer. The sugar beet sequence was fragmented into short *k*-mers of $k$=17 in length and we queried for exact matches in the NCBI fungal ITS database. The chosen primer was a 17-nucleotide sequence with no significant similarity to any NCBI fungal ITS sequences and an annealing temperature ~10°C higher than the ITS1f-Kyo2 primer, whose extension it would block. The PNA blocking primer was at 0.4 µM concentration in a 25 µL PCR. PCR products were purified using SureClean (Bioline, London, UK). Barcodes were attached to each of 11 DNA samples per run using the Rapid PCR Barcoding kit (Oxford Nanopore Technologies) and

LongAmp Taq 2X master mix (New England BioLabs, MA, USA) according to the Oxford Nanopore Technologies protocol. Barcoded amplicon libraries were purified with AMPure XP magnetic beads (Beckman Coulter, CA, USA) and resuspended in 10 µL of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. Barcoded libraries were pooled in equimolar concentrations to a total of ~100 ng, then loaded onto a MinION flow cell (R9.4.1) according to the manufacturer's instructions. MinKNOW software (Oxford Nanopore Technologies) was used to execute sequencing, and raw reads (fast5) were accumulated over 48 h with live base-calling (fast option) to output fastq files. Reads were demultiplexed in real-time using MinKNOW to output reads into a separate directory per barcode.

The downstream processing of data was performed largely as established by Mafune et al. (2019) using their python scripts available at https://github.com/mycoophile/nanopore-ITS. Fastq files were filtered through NanoFilt v2.6.0 (De Coster et al. 2018) for a Q-score of 10. Since the following pairwise sequence alignment step requires considerable memory and processing power, we randomly selected 5,000 sequences per barcode to analyze, using the sample function of seqtk (Li 2013). Sequences were aligned per barcode using the global pairwise alignment option, -gins1 in MAFFT v7.402 with reduced gap penalties using options –op 0.5 and –gop 0.5 (Katoh and Standley 2013). Seqret (part of EMBOSS suite of tools) (Rice et al. 2000) was used to convert the fasta alignment files to phylip format using the -osformat phylip option. A distance matrix was calculated for each alignment using the F84 model in PHYLIP v3.697 (Felsenstein 2004) dnadist with default parameters. Operational taxonomic unit (OTU) clustering was performed using the OptiClust method in Mothur v1.44.1 (Schloss et al. 2009) using 92% sequence similarity (cut-off = 0.08). OTUs were organized into clusters for subsequent MAFFT alignment (same options as before) using the fasta_otu_collater2.py script

(Mafune et al. 2019). OTUs with less than 10 sequences were removed from further analysis, since BLASTn searches of consensus sequences from small OTUs tended to give uncertain results at the genus level. OTUs were aligned using MAFFT and an ungapped consensus sequence produced from the alignment using the OTU_UnGapCons_v4.py script. The consensus sequences were used to perform BLASTn alignments of the NCBI database, and taxon identities were assigned using the top hits. The number of reads assigned to a particular taxon was converted to relative abundance (%) by calculating the percentage of total classified reads represented by that taxon. The mean relative abundance of each identified taxon over three biological replicates was calculated per seed lot. The resulting graphs were generated using the ggplot2 package (Wickham et al. 2016) in RStudio (Team 2015).

## 3.4. Results

### 3.4.1. Baiting and identification of *C. beticola* from sugar beet seed

To initially assess whether sugar beet seed harbored *C. beticola*, a minimum of 50 seeds from each of 37 sugar beet seed lots were placed on PDA. After 7 to 10 days, fungal growth suggestive of *C. beticola* was identified in ten seed lots (Fig. 3.1). Fungal growth was initiated from either the seed itself, pelleted (Fig. 3.1A) or unpelleted (Fig. 3.1B), or the hypocotyl of a germinated seed (not shown).

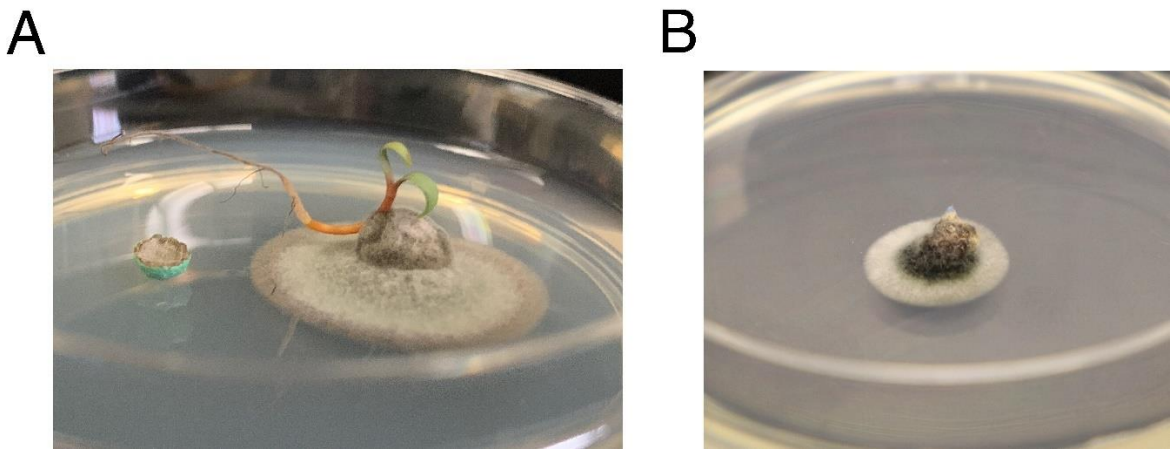


Figure 3.1. Fungal growth from surface-sterilized sugar beet seed. Fungal growth was either directly from pelleted seed A, or from unpelleted seed B, and was later confirmed to be *Cercospora beticola* using species-specific qPCR and Sanger sequencing.

Using species-specific qPCR of the calmodulin gene in fungal isolates, 38 *C. beticola* isolates were confirmed from ten seed lots (Table 3.1). Since we were able to obtain multiple isolates from several seed lots, we questioned whether specific *C. beticola* genotypes may be particularly adapted for the ability to remain viable on sugar beet seed and thus would be over-represented within a seed lot. To help assess this, SSR markers were run on all 38 seed-borne *C. beticola* isolates. These analyses indicated that there were no redundant genotypes identified in this study (Table 3.1).

Table 3.1. SSR genotypes and fungicide sensitivity of *Cercospora beticola* isolates baited from 37 sugar beet seed lots. A total of 38 *C. beticola* isolates grew from 10 of the 37 seed lots. Each isolate underwent SSR genotyping and were assayed for DMI fungicide sensitivity (tetraconazole $EC_{50}$ value measurements) and for the presence of the G143A QoI fungicide resistance mutation (qPCR).

| Seed lot number | Isolate strain number | SSR genotype[2] | Tetraconazole $EC_{50}$[3] | QoI sensitivity[4] |
|---|---|---|---|---|
| 1 | NA[1] | | | |
| 2 | 20-S066 | AA | 68.897 | R |
| | 20-S075 | AB | 40.080 | R |
| 3 | 20-S011 | AC | 6.116 | R |
| | 20-S012 | AD | 14.773 | R |
| | 20-S013 | AE | 26.829 | R |
| | 20-S014 | AF | 1.739 | R |
| | 20-S015 | AG | 21.250 | R |
| | 20-S016 | AH | 16.124 | R |
| 4 | NA | | | |
| 5 | 20-S001 | AI | 0.955 | R |
| | 20-S052 | AJ | 0.766 | R |
| 6 | 20-S004 | AK | 5.288 | R |
| | 20-S005 | AL | 63.095 | R |
| | 20-S006 | AM | 11.765 | R |
| | 20-S007 | AN | 26.108 | R |
| | 20-S008 | AO | 5.173 | R |
| | 20-S009 | AP | 21.964 | R |
| | 20-S010 | AQ | 0.541 | R |
| 7 | NA | | | |
| 8 | NA | | | |
| 9 | 20-S067 | AR | 0.782 | R |
| 10 | 20-S019 | AS | 7.794 | R |
| | 20-S020 | AT | 5.814 | R |
| | 20-S021 | AU | 68.036 | R |
| | 20-S022 | AV | 10.000 | R |
| | 20-S023 | AW | 62.063 | R |
| | 20-S024 | AX | 51.636 | R |
| | 20-S025 | AY | 67.363 | R |
| | 20-S026 | AZ | 23.991 | R |
| | 20-S027 | BA | 0.667 | R |
| | 20-S028 | BB | 77.734 | R |
| | 20-S029 | BC | 3.714 | R |
| | 20-S030 | BD | 26.254 | R |
| | 20-S036 | BE | 86.309 | R |
| | 20-S065 | BF | 26.720 | R |
| | 20-S035 | BG | 45.848 | R |
| 11 | NA | | | |
| 12 | 20-S038 | BH | 46.595 | R |

Table 3.1. SSR genotypes and fungicide sensitivity of *Cercospora beticola* isolates baited from 37 sugar beet seed lots (continued). A total of 38 *C. beticola* isolates grew from 10 of the 37 seed lots. Each isolate underwent SSR genotyping and were assayed for DMI fungicide sensitivity (tetraconazole $EC_{50}$ value measurements) and for the presence of the G143A QoI fungicide resistance mutation (qPCR).

| Seed lot number | Isolate strain number | SSR genotype[2] | Tetraconazole $EC_{50}$[3] | QoI sensitivity[4] |
|---|---|---|---|---|
| 13 | NA | | | |
| 14 | 20-S040 | BI | 53.095 | R |
| | 20-S046 | BJ | 7.604 | R |
| 15 | NA | | | |
| 16 | NA | | | |
| 17 | NA | | | |
| 18 | 20-S074 | BK | 25.53 | R |
| 19 | NA | | | |
| 20 | NA | | | |
| 21 | NA | | | |
| 22 | NA | | | |
| 23 | NA | | | |
| 24 | 20-S031 | BL | 0.422 | R |
| 25 | NA | | | |
| 26 | NA | | | |
| 27 | NA | | | |
| 28 | NA | | | |
| 29 | NA | | | |
| 30 | NA | | | |
| 31 | NA | | | |
| 32 | NA | | | |
| 33 | NA | | | |
| 34 | NA | | | |
| 35 | NA | | | |
| 36 | NA | | | |
| 37 | NA | | | |

[1] Not applicable (NA) indicates that no *C. beticola* strain was identified from this seed lot.
[2] See Materials and methods for simple sequence repeat (SSR) genotype methodology.
[3] Tetraconazole $EC_{50}$ values calculated as described by Secor and Rivera (2012).
[4] QoI resistance was assessed as described by Bolton et al. (2013).

### 3.4.2. Fungicide resistance profiles of *C. beticola* from infested sugar beet seed

We assayed 38 *C. beticola* isolates derived from sugar beet seed for resistance to both QoI and DMI fungicide classes. Real-time PCR methodology to detect the G143A mutation conferring resistance to QoI fungicides (Bolton et al. 2013) revealed that all isolates in this study contained this mutation and were therefore considered QoI-resistant (Table 3.1). For tetraconazole, six isolates had $EC_{50}$ values below 1.0 µg/mL and were considered DMI-sensitive (Bolton et al. 2012). The remaining 32 isolates had $EC_{50}$s over 1.0 µg/mL, demonstrating that the majority had reduced sensitivity to DMI fungicides.

### 3.4.3. *C. beticola* seed-to-seedling transmission efficiency

To determine whether seed-borne *C. beticola* could initiate disease in sugar beet, we planted pelleted sugar beet seed from seed lots 1, 3, and 10 and observed developing plants for CLS symptoms. Seed lot 1 was chosen because no *C. beticola* was recovered from this variety (Table 3.1) and, therefore, it acted as a negative control. In contrast, we were successful in baiting several *C. beticola* isolates from seed lots 3 and 10 (Table 3.1). Sugar beet plants were observed for 15 weeks. After 15 weeks, 54 lesions caused by *C. beticola* were identified among the 12 plants in seed lot 3, with an average of 4.5 lesions per plant. Likewise, 132 lesions caused by *C. beticola* were identified among the 12 plants in seed lot 10, with an average of 11 lesions per plant. No CLS lesions were observed on plants sown from seed lot 1. Examples of sugar beet leaves harboring CLS lesions from seed lot 10 are shown in Figure 3.2. Fungal isolates were recovered from a minimum of 5 lesions per seed lot. All recovered isolates were confirmed to be *C. beticola* using the species-specific qPCR described by Knight et al (2020). The experiment was repeated with similar results.

Figure 3.2. Cercospora leaf spot lesions on sugar beet that developed from seed-borne *Cercospora beticola*. Harvested leaves were 13 weeks old and were taken from plants that developed from seed lot 10.

### 3.4.4. Xylem sap analysis

To improve our understanding of how *C. beticola* infects sugar beet leaves via seedborne inoculum, we planted pelleted sugar beet seed from seed lots 1, 3, and 10. After five weeks of growth, the hypocotyl was cut approximately 1 cm above the sugar beet root and xylem sap was collected from the cut surface. *C. beticola*-specific qPCR primers for the calmodulin gene were used to screen DNA isolated from pooled xylem sap for the presence of this pathogen. We obtained PCR amplicons only from seed lot 10 in both experimental repeats. To ensure that the obtained amplicons were from *C. beticola*, PCR products were sequenced, and the resulting sequences were used as queries at GenBank. All amplicons were 100% matches to *C. beticola*.

### 3.4.5. Profiling of seed-associated fungi using MinION sequencing

Because we isolated several fungal species in addition to *C. beticola* from sugar beet seed, we were interested in developing an inventory of the fungal species derived from selected seed lots. To that end, we performed long-read sequencing of ITS amplicons generated from DNA extracted from eleven selected seed lots (1, 3, 5, 6, 8, 10, 19, 24, 27, 29 and 30 in Table 3.1). The amplicons, spanning the entire ITS region, were sequenced using the MinION sequencer which uses nanopore long-read sequencing technology.

In order to specifically reduce the unintended amplification of sugar beet ITS sequence, we designed a peptide nucleic acid (PNA) blocking primer for use in the initial ITS PCR. PNAs can be used to reduce amplification of complementary DNA sequences by forming PNA-DNA duplexes, which are not recognized by DNA polymerases and cannot act as primers in PCR (von Wintzingerode *et al.* 2000). PNAs can be designed to inhibit amplification of undesired sequences, either by competitive DNA clamping with the forward or reverse primers, or by elongation arrest. In this study, the PNA primer was a 17-nucleotide sequence designed as an elongation arrest blocking primer within the ITS amplicon, in a region unique to sugar beet when compared to fungal sequences. PCRs were performed for seed lot 1 DNA (biological replicate #1), both with and without the blocking primer, and its presence reduced the relative abundance of *Beta vulgaris* reads substantially from 72.5% to 3.8% (Fig. 3.3).

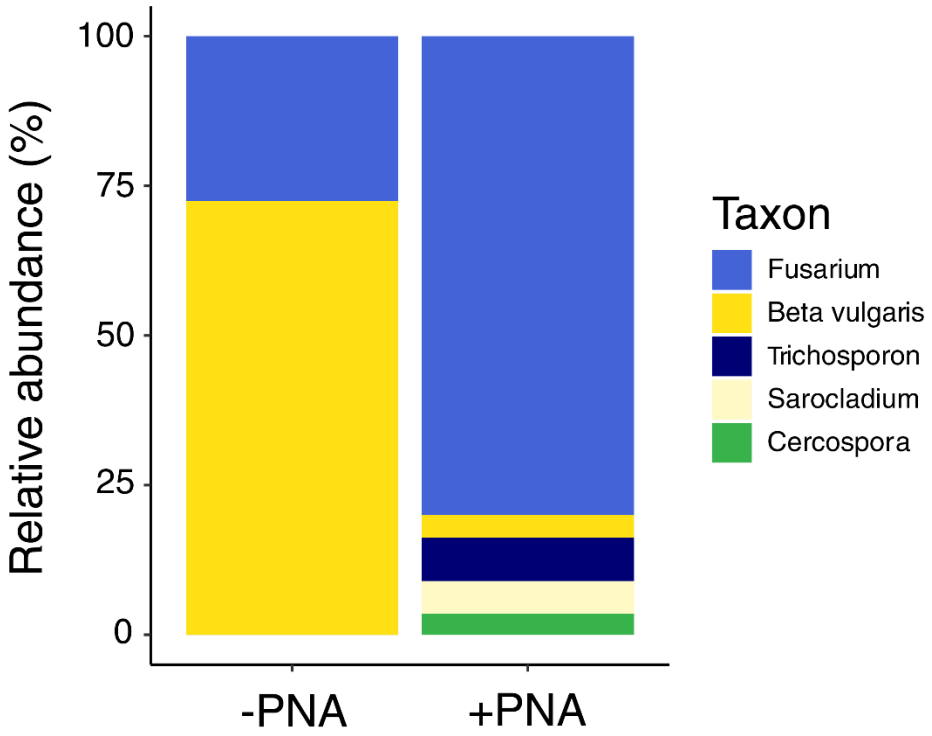


Figure 3.3. Relative abundance of taxa identified through MinION sequencing of ITS amplicons in the same biological sample (seed lot 1, biological replicate #1) both without (-) and with (+) a peptide nucleic acid (PNA) blocking primer included in the initial ITS PCR. The PNA blocking primer was designed to specifically inhibit amplification of sugar beet ITS sequence. The relative abundance is the proportion of total classified reads (%) assigned to a specific taxon.

We accumulated enough reads (from ~20K to ~150K per sample) for downstream analyses in under 24 h for 11 barcoded samples loaded simultaneously. We first attempted to utilize the "What's In My Pot" (WIMP) workflow (Juul et al. 2015), but found it was inappropriate for classifying reads of this length (~1kb) and often misclassified individual reads when compared to manual BLASTn analysis (not shown). Instead, we used a pipeline established by Mafune et al. (2019) for identifying fungal species from ITS amplicons sequenced with a MinION. This strategy derives highly accurate consensus sequences to overcome the high error rate of nanopore sequencing. After read filtering, read clustering, and BLAST analysis of operative taxonomic units (OTUs), we could confidently classify OTUs to the genus level.

To demonstrate that the sequencing method was robust with high reproducibility for a single biological sample (Fig. 3.4A), we simultaneously set up three ITS PCRs from the same DNA sample (seed lot 19, biological replicate #1) and performed MinION sequencing. The relative abundance of each species identified was highly consistent between each technical replicate, with the highest difference being in *B. vulgaris* reads at a maximum of ~4%. This could be attributed to varying efficacy of the sugar beet specific ITS blocking primer between PCRs, used to inhibit amplification of sugar beet ITS sequences. We also demonstrated that the sub-sampling of 5,000 reads in the downstream pipeline was sufficient to capture a reliable representation of the fungal profile. Three different sub-samples of 5,000 reads were extracted from the same sequenced sample and downstream analysis produced near-identical results (Fig. 3.4B).

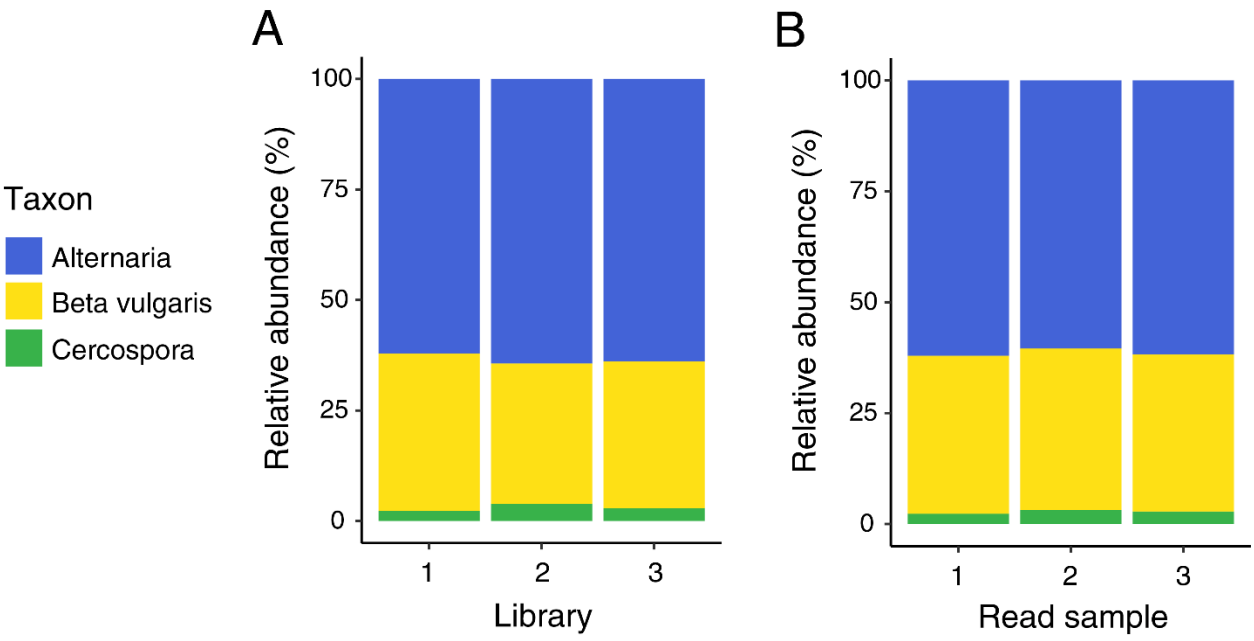


Figure 3.4. A, Demonstration of reproducibility of the MinION amplicon sequencing method by sequencing three different PCRs from a single biological sample and B, taking three different sub-sets of 5,000 reads for downstream analysis from a single sequencing run. The relative abundance is the proportion of total classified reads (%) assigned to a specific taxon.

The mean proportion of reads classified as different taxonomic groups are shown for each of the 11 seed lots (Fig. 3.5). In total, we identified 19 different fungal genera present on sugar beet seed. In all seed lots except 3 and 4, *Fusarium* was the most highly represented fungal genus. *Alternaria* was the second most represented genus and made up an average of 89% of classified reads in seed lot 5. *Cercospora* was the third most prevalent genus throughout seed lots.

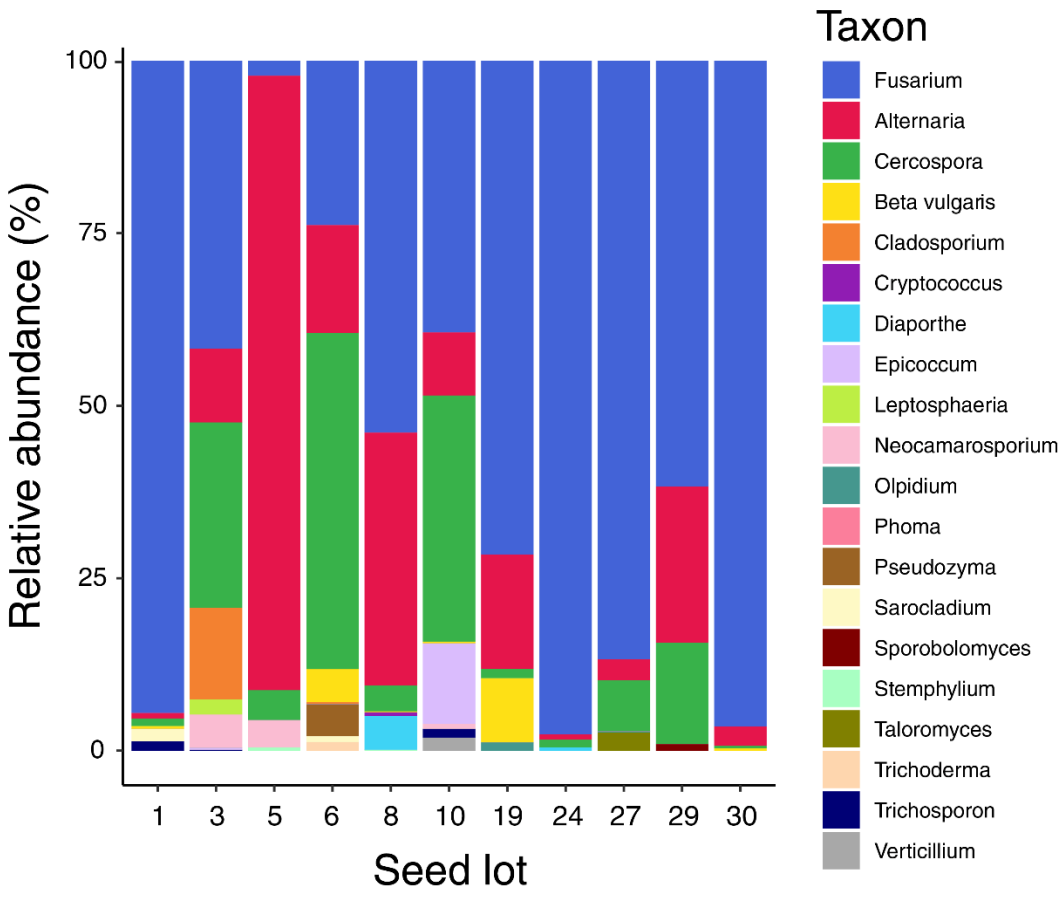


Figure 3.5. Relative abundance of taxa identified in 11 sugar beet seed lots through MinION sequencing of ITS amplicons**.** The relative abundance is the proportion of total classified reads (%) assigned to a specific taxon and is a mean value of three different biological samples from each seed lot.

Reads matching to members of the *Cercospora* genus were found in every single seed lot tested, although the relative abundance varied a lot from 0.4 to 48.7% (Fig. 3.5, 3.6). Seed lots 3,

6 and 10 had the largest relative abundance of *Cercospora* reads and also the largest number of *C. beticola* isolates baited from seed (Fig. 3.6). Likewise, seed lots 1, 8, 19, 27, 29 and 30 had no *C. beticola* growth on plates while also exhibited lower relative abundance of *Cercospora* reads. There was a higher variability in relative abundance between biological replicates for seed lots harboring more *C. beticola* (Fig. 3.6).

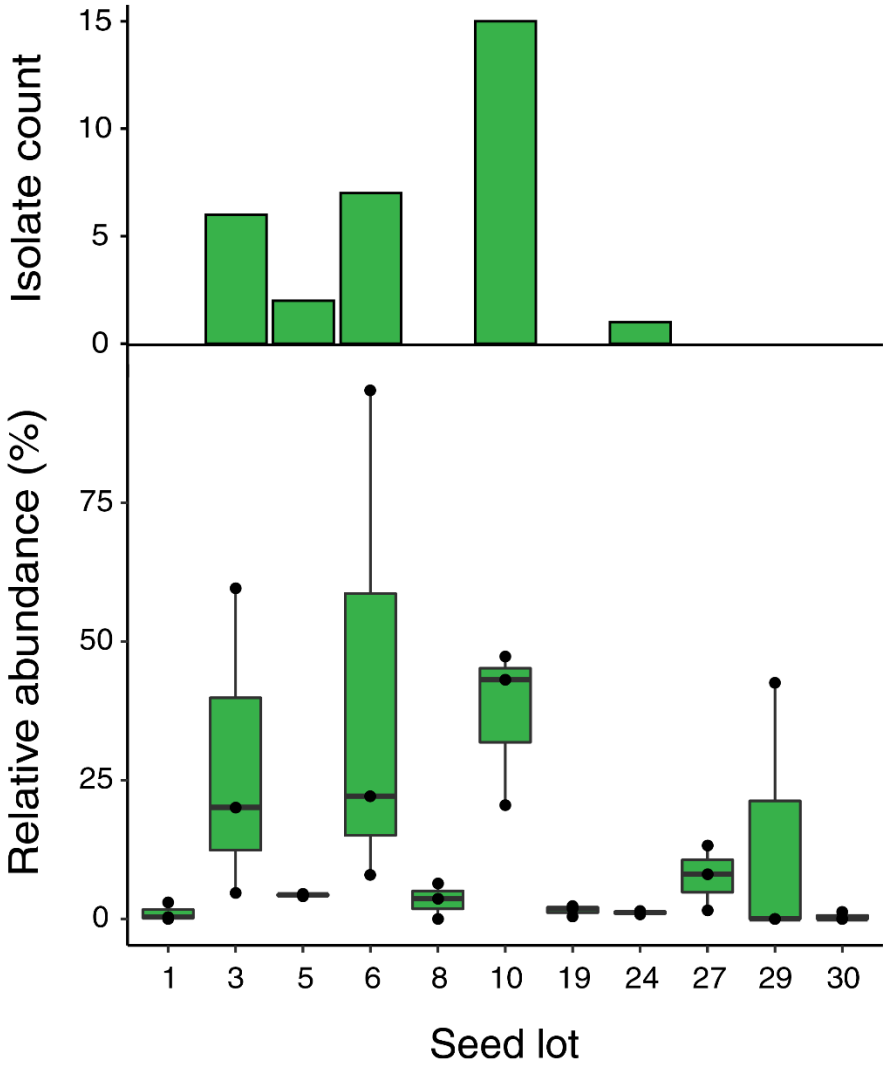


Figure 3.6. The number of *C. beticola* isolates baited from 11 sugar beet lots (upper panel) and the box and whiskers plots showing variation in relative abundance of *Cercospora* (%) identified through MinION sequencing of ITS amplicons amongst three biological replicates (lower panel). The relative abundance is the proportion of total classified reads (%) assigned to the *Cercospora* genus.

DNA from all seed lots was extracted, amplified, and sequenced in three separate batches, representing the three biological replicates. We noticed consistent differences for different biological replicates throughout seed lots e.g. the highest proportion of unclassified reads for each seed sample tended to be in biological replicate #3 (Table 3.2). This suggests that the procedure may be sensitive to variability between DNA extractions, individual PCR setups and/or library preparations despite consistent parameters being used.

Table 3.2. Percentage of reads assigned to each taxonomic group via ITS amplicon sequencing using the MinION in three different biological samples from each of 11 seed lots.

| | Biological Replicate #1 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Seed Lot | 1 | 3 | 5 | 6 | 8 | 10 | 19 | 24 | 27 | 29 | 30 |
| *Fusarium* | 46.54 | 4.8 | 0 | 28.76 | 7.34 | 4.64 | 0 | 51.24 | 45.86 | 33.14 | 56.46 |
| *Alternaria* | 0 | 1.96 | 50.48 | 18.5 | 32.2 | 3.6 | 24.76 | 0.66 | 1.76 | 1.1 | 0 |
| *Cercospora* | 1.62 | 31.92 | 2.22 | 4.16 | 1.84 | 25.62 | 0.92 | 0.44 | 7.24 | 25.34 | 0 |
| *Neocamarosporium* | 0 | 8.1 | 2.08 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Leptosphaeria* | 0 | 3.66 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* | 0 | 2.44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Phoma* | 0 | 0.42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichosporon* | 2.3 | 0.24 | 0 | 0 | 0 | 1.96 | 0 | 0 | 0 | 0 | 0 |
| *Sarocladium* | 3.04 | 0 | 0 | 1.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Diaporthe* | 0 | 0 | 0 | 0 | 8.08 | 0 | 0 | 0.8 | 0 | 0 | 0 |
| *Cryptococcus* | 0 | 0 | 0 | 0 | 0.92 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Epicoccum* | 0 | 0 | 0 | 0 | 0 | 18.38 | 0 | 0 | 0 | 0 | 0 |
| *Stemphylium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Taloromyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Verticillium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sporobolomyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Olpidium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pseudozyma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichoderma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Beta vulgaris* | 0.4 | 0 | 0 | 0 | 0.22 | 0 | 14.2 | 0 | 0 | 0 | 0.56 |
| Unclassified | 46.1 | 46.46 | 45.22 | 47.56 | 49.4 | 45.8 | 60.12 | 46.86 | 45.14 | 40.42 | 42.98 |

Table 3.2. Percentage of reads assigned to each taxonomic group via ITS amplicon sequencing using the MinION in three different biological samples from each of 11 seed lots (continued).

| | Biological Replicate #2 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Seed Lot | 1 | 3 | 5 | 6 | 8 | 10 | 19 | 24 | 27 | 29 | 30 |
| *Fusarium* | 66.2 | 35.32 | 1.52 | 0 | 41 | 39.96 | 66.08 | 66.78 | 53.72 | 46 | 61.56 |
| *Alternaria* | 1.34 | 5.44 | 61.64 | 0.64 | 23.36 | 9.08 | 0 | 0 | 1.14 | 18.04 | 2.12 |
| *Cercospora* | 0.22 | 2.96 | 2.98 | 52.1 | 4.4 | 12.68 | 0.26 | 0.76 | 0.9 | 0 | 0 |
| *Neocamarosporium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Leptosphaeria* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* | 0 | 19.76 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Phoma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichosporon* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sarocladium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Diaporthe* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cryptococcus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Epicoccum* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Stemphylium* | 0 | 0 | 0.26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Taloromyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.2 | 0 | 0 |
| *Verticillium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sporobolomyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.62 | 0 |
| *Olpidium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pseudozyma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichoderma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Beta vulgaris* | 0 | 0 | 0 | 1.98 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 32.24 | 36.52 | 33.6 | 45.28 | 31.24 | 38.28 | 33.66 | 32.46 | 40.04 | 34.34 | 36.32 |

Table 3.2. Percentage of reads assigned to each taxonomic group via ITS amplicon sequencing using the MinION in three different biological samples from each of 11 seed lots (continued).

| | Biological Replicate #3 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Seed Lot | 1 | 3 | 5 | 6 | 8 | 10 | 19 | 24 | 27 | 29 | 30 |
| *Fusarium* | 45.98 | 29.72 | 1.96 | 0.44 | 41.22 | 17.32 | 42.88 | 46.04 | 40.56 | 27.22 | 45.4 |
| *Alternaria* | 0 | 10.5 | 29.44 | 0 | 5.28 | 1.68 | 0.52 | 0.62 | 2.1 | 19.78 | 2.64 |
| *Cercospora* | 0 | 10.12 | 1.64 | 3.38 | 0 | 17.8 | 0.86 | 0.68 | 3.78 | 0 | 0.6 |
| *Neocamarosporium* | 0 | 0 | 4.26 | 0 | 0 | 1.12 | 0 | 0 | 0 | 0 | 0 |
| *Leptosphaeria* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* | 0 | 0 | 0 | 0.36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Phoma* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichosporon* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sarocladium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Diaporthe* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cryptococcus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Epicoccum* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Stemphylium* | 0 | 0 | 0.48 | 0 | 0.24 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Taloromyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Verticillium* | 0 | 0 | 0 | 0 | 0 | 3.02 | 0 | 0 | 0 | 0 | 0 |
| *Sporobolomyces* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Olpidium* | 0 | 0 | 0 | 0 | 0 | 0 | 1.8 | 0 | 0.32 | 0 | 0 |
| *Pseudozyma* | 0 | 0 | 0 | 5.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Trichoderma* | 0 | 0 | 0 | 1.54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Beta vulgaris* | 0.24 | 0 | 0 | 3.86 | 0 | 0.34 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 53.78 | 49.66 | 62.22 | 84.72 | 53.26 | 58.72 | 53.94 | 52.66 | 53.24 | 53 | 51.36 |

## 3.5. Discussion

Seed-borne phytopathogens present a huge threat to agriculture in today's global trade scenario. By surviving in seed, pathogens can be widely distributed by humans across natural boundaries and can be introduced into new areas (Kumar 2020). The long-distance movement of *C. beticola* has been evidenced indirectly in several recent population genetic studies through the identification of recurrent clonal lineages across continents (Knight et al. 2019b; Vaghefi et al. 2017c). Spatial and temporal shifts in field genotypes of *C. beticola* have also been associated with the use of external seed sources (Knight et al. 2018; Vaghefi et al. 2017a). Furthermore, much anecdotal evidence has long suggested the presence of seed-borne *C. beticola* and its association with CLS epidemics in sugar beet (Jacobsen and Franc 2009; Richardson and Noble 1968; Schürnbrand 1952; Vereijssen et al. 2004).

In this study, we identify viable *C. beticola* in multiple sugar beet seed lots and demonstrate the ability of seed-borne *C. beticola* to act as primary inoculum and initiate disease. Similar findings were presented by McKay and Pool (1918), but no other study has provided direct evidence for seed transmission in this pathosystem for over 100 years. Seed transmission of *C. beticola* was demonstrated for table beet by Knight et al. (2020), who found that seed-borne *C. beticola* could cause CLS in seedlings.

As an initial indication of the presence of *C. beticola* in sugar beet seed, we attempted to bait the fungus using plate growth assays. Ten of the 37 sugar beet seed lots tested had fungal growth identified as *C. beticola*. In addition to *C. beticola*, several other fungal and bacterial species were also identified (not shown). In fact, the abundance of microflora in sugar beet seed often increased the amount of time necessary to purify *C. beticola* from other fungal species in baiting studies. Moreover, *C. beticola* is notoriously slow-growing in culture which can impede

its detection when competing with other fungi (Khan et al. 2008; Knight et al. 2020). Consequently, the targeted and sensitive method of detecting *C. beticola* by qPCR, as described by Shrestha et al. (2020) and Knight and Pethybridge (2020), is useful for sequence-specific detection of this pathogen.

Historically, the cultivation of fungi has been critical for detection and identification based on morphology. However, this can limit identification to culturable fungi that are present in relatively high abundance and/or are faster growing than the species of interest (Huffnagle and Noverr 2013). Because we were also interested in obtaining a comprehensive overview of the fungal species diversity present in sugar beet seed, we sequenced the full-length *ITS1* and *ITS2* rRNA regions from seed DNA using the MinION nanopore sequencing platform. Analysis of sequencing reads identified amplicons from the *Cercospora* genus in each of the 11 seed lots tested but relative abundance ranged from 0.4 to 48.7%. The relative abundance of *Cercospora* reads largely correlated with the amount of *C. beticola* baited from seed. We identified *Cercospora* reads in seed lots that failed to bait *C. beticola* in plate growth assays, supporting the potential for rapid in-house nanopore-based sequencing to detect fungal contamination in seed batches that otherwise stay undetected. It is also possible that these seed batches harbor unviable *C. beticola,* and that thus do not represent potential risks for crop production and this should be studied in more depth in the future.

To investigate seed-to-seedling transmission of *C. beticola*, we planted pelleted seed from two seed lots that were infested with *C. beticola* and seed lot 1 that did not appear to harbor viable *C. beticola* because we were unable to grow the fungus from this seed. The plants that developed from the infested seed lots developed CLS symptoms, whereas the seed lot that was not apparently infested lacked any symptoms during the period of observation used in this study.

The chambers used in this study had not been used previously for sugar beet growth or associated *C. beticola* inoculations. Nonetheless, all chambers were thoroughly sanitized prior to seed to seedling experiments to ensure that no *C. beticola* spores were present inside the chambers that may have inadvertently initiated disease and provided false-positive results. Given these safeguards and the resulting disease, specifically in seed lots 3 and 10, our results strongly suggest that seed-borne *C. beticola* can initiate disease in sugar beet.

During the transmission assays, we detected the presence of *C. beticola* in xylem sap via species-specific qPCR, suggesting that the fungus may utilize the vascular system to spread upwards through the sugar beet plant to the foliage. Seed lot 10 developed the highest number of lesions per plant (11) and *C. beticola* was detected in xylem sap. However, seed lot 3 developed CLS lesions (4.5 per plant) and *C. beticola* was not detected in the xylem. It was previously demonstrated that root infection by *C. beticola* can give rise to leaf symptoms (Vereijssen et al. 2004; Vereijssen et al. 2005) and it was deemed unlikely to have occurred through epiphytic growth due to the low frequency of stem lesions. Further microscopic and molecular studies are required to establish precisely how *C. beticola* spreads from the seed to initiate foliar disease. Thirty-eight *C. beticola* isolates isolated from sugar beet seeds were tested for sensitivity to both QoI and DMI fungicides. All isolates tested harbored the G143A mutation in the *cytb* gene, which is associated with resistance to QoI fungicides (Bolton et al. 2013). Isolates tested for DMI sensitivity generally had high $EC_{50}$ values (>1 µg/mL), and hence were considered DMI-resistant (Bolton et al. 2012b). Taken together, these data suggest these isolates originated from growing areas where fungicides are routinely used, which drives selection for resistant strains in the population. It is also important to consider that the movement of these strains via seed could facilitate the spread of fungicide resistance across continents, reducing efficacy of current

fungicide chemistries at a faster rate. Ideally, sugar beet seed would be produced in areas where *C. beticola* does not thrive and disease is rare. However, if this cannot be the case, chemical seed treatments may need to be considered to manage *C. beticola* in future. Such seed treatments should be from effective FRAC groups with low risk of resistance development. In the current study, *C. beticola* grew directly from seed from ten different commercial seed lots (European and US) while pelleted seed from two commercial cultivars exhibited seed-to-seedling transmission. Therefore, current seed treatments may not be effective in managing seed-borne *C. beticola*.

Most importantly, we have identified a primary source of inoculum that should be considered in integrated management programs of CLS in sugar beet. The dynamics of seed-borne inoculum in field-based epidemics should be investigated further. For example, it is currently not known what quantity of inoculum is required to initiate an epidemic. Further studies looking at the host and environmental factors affecting seed-to-seedling transmission rates will need to be carried out. Since CLS is a polycyclic disease, it is likely that very few instances of seed-to-seedling transmission are required in a field to initiate a severe epidemic, granted that the host is susceptible and environmental conditions are conducive (Knight et al. 2020). Rapid, sensitive and accurate seed testing procedures should be implemented to be able to detect *C. beticola* at a sufficient threshold to initiate disease. Seed may need to be routinely tested and certified in the future to demonstrate that it is free of *C. beticola* inoculum. To remove this source of inoculum, sugar beet seed may need to be treated. The choice of treatment may depend on which part of the seed that *C. beticola* inhabits. The fungus has been suggested to be endophytic and perhaps be present in the cork or close to the embryo (Vereijssen et al. 2004). Pathogens that colonize internal seed tissues may require physical treatment such as hot water or systemic chemicals that can translocate in the seed (Taylor and Harman 1990). Soaking seed in

formaldehyde was demonstrated by McKay and Pool (1918) to reduce *C. beticola* inoculum but the efficacies of modern fungicidal seed treatments are yet to be explored.

To our knowledge, this is the first study that investigates the fungal microbiome of sugar beet seed. Intriguingly, we find the presence of multiple, potentially viable, fungal pathogens in addition to *C. beticola* in sugar beet seed. We surface-sterilized seed and removed seed pellets/coats prior to DNA extraction, and so any epiphytic surface colonizers of seed should have been eliminated. Sequencing of ITS amplicons using the MinION was a fast, reproducible and cost-effective in-house method to identify dominant fungal species present on the seed. The three most abundant taxa found were *Fusarium*, *Alternaria* and *Cercospora* and were identified on every seed lot tested. On average, these three genera made up 93% of relative abundance in seed lots but the composition varied widely between lots. In total, we identified 19 different fungal genera present on sugar beet seed via long-read sequencing. Many of these were "rare" and only detected in one or two seed lots. In addition to *Cercospora*, some of these genera contain known pathogens of sugar beet, such as *Fusarium oxysporum* f. sp. *betae* causing Fusarium yellows (Webb et al. 2019), *Fusarium secorum* causing Fusarium yellowing decline (Secor et al. 2014), various *Alternaria* spp. causing Alternaria leaf spot, *Neocamarosporium betae* causing Phoma leaf spot and root rot (Vaghefi et al. 2019), *Stemphylium* spp. causing Yellow leaf spot (Hanse et al. 2015) and *Verticillium dahliae* causing Verticillium wilt (Karadimos et al. 2000). *Sarocladium* and *Talaromyces* spp. were both isolated from root lesions during sugar beet storage (Strausbaugh et al. 2015). Further experiments will be needed to determine if any of these species are viable on seed and can cause disease in seed-to-seedling assays. *N. betae* is already known to be a seed-borne pathogen of sugar beet (Edson 1915; Herr 1971). *Alternaria* and *Fusarium* spp. often cause diseases as seed-borne inoculum (Blanco and

Aveling 2018; Groves and Skolko 1944). Two seed lots in this study also contained reads classified as *Olpidium*, a genus containing the chytrid fungus *O. brassicae* which can vector Beet black scorch virus (BBSV) (Junxi et al. 1999). Since *Olpidium* spp. are obligate biotrophs, they cannot be cultured *in vitro* and so sequencing is the only feasible option to determine their presence (Sekimoto et al. 2011). Additional sequencing analyses will need to be performed to determine if BBSV is also present.

Several yeast genera were identified on sugar beet seed: *Cryptococcus*, *Pseudozyma*, *Sporobolomyces*, and *Trichosporon*. *Cryptococcus* and *Sporobolomyces* were previously identified as predominant fungal members of the sugar beet phyllosphere, along with *Cladosporium* and *Alternaria* (Thompson et al. 1993). Some naturally-occurring fungal species have the ability to suppress pathogenic fungi, which could be harnessed for biocontrol. *Trichoderma* and *Talaromyces* spp. were each found to be present in single sugar beet seed lots, and have previously been tested experimentally as biocontrol agents (Kakvan et al. 2013; Naraghi et al. 2014).

Long-read ITS amplicon sequencing using the MinION is a rapid and cost-effective method for profiling microbial communities (Benítez-Páez et al. 2016; Calus et al. 2018; Kerkhof et al. 2017; Kilianski et al. 2015; Mafune et al. 2019). In our study, we were able to confidently assign OTUs to the genus level. Several of the genera we described are known for having identical ITS sequences shared amongst different species, such as *Fusarium* (O'Donnell and Cigelnik 1997), and may require additional markers to define species. When trying to quantify the abundance of different taxa, ITS amplicon sequencing can result in over- or under-representation of certain groups (Loit et al. 2019; Mafune et al. 2019). This is likely due to the variable number of repeats of rRNA genes in different fungal species (Schoch et al. 2012) and

could also be due to PCR biases (Bellemain et al. 2010). For example, in this study we identified *Fusarium* spp. to have the highest number of fungal reads in multiple seed lots, but this may not reflect the real relative abundance in terms of fungal biomass. To overcome this, a community standard can be included as described by Taylor et al. (2016), Benítez-Páez and Sanz (2017), Bakker (2018), and Mafune et al. (2019). Inclusion of a community standard for each sequencing run may also help to normalize variation in relative abundance between experiments. This particular method and pipeline also failed to confidently identify small OTUs with 10 or less reads, likely from rare taxa. This was likely due to the high error rate of nanopore sequencing but also due to the limited number of reads (5,000) analyzed simultaneously because of the initial resource-intensive global pairwise alignment step. Future improvements in nanopore chemistry to minimize base-calling errors could overcome some of these issues. Moreover, the development of new software specifically for aligning and clustering MinION reads of amplicons derived from complex communities would be helpful.

### 3.6. Conclusion

In summary, we provide direct evidence for the ability of seed-borne *C. beticola* to initiate CLS in sugar beet. We propose that sugar beet seed should be considered as a primary inoculum source in the management of CLS in the future. New management strategies may include routine seed testing for the presence of *C. beticola* and treatment of seed to reduce the fungal density. This was the first study to investigate the fungal microbiome of sugar beet seed and we achieved this through long read ITS amplicon sequencing with the MinION sequencer. We identified the presence of other potentially viable seed-borne fungi from multiple genera that harbor common sugar beet pathogens, including *Alternaria* and *Fusarium*. Future investigations will clarify whether the seed is also a source of inoculum for other important diseases of sugar beet.

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